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OF SODIUM TRANSPORT
IN RAT MYOMETRIUM

UNIVERSITY ALBERTA

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ULTRASTRUCTURAL ASPECTS OF SODIUM TRANSPORT IN RAT MYOMETRIUM

BY

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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EDMONTON, ALBERTA
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Ultrastructural Aspects of Na^+ Transport in Rat Myometrium, submitted by Robert E. Garfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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TO JOSETTE, JOHN AND PATRICIA

Abstract

Membrane vesicles have been proposed as sites for volume control in rat uterine smooth muscle (Daniel and Robinson, 1971c). To test the vesicular hypothesis, the ultrastructure and Na^+, K^+, ATP and H_2O contents of rat uterine smmoth muscle were examined after various treatments with metabolism and transport inhibitors. Metabolic inhibition with iodoacetate (IAA) plus dinitrophenol (DNP) or IAA alone produced ATP depletion and reduced the number of membrane vesicles with associated changes in cations and water contents. Effects of DNP alone and ethacrynic acid were also studied. Inhibition of Na^+-K^+-ATPase with ouabain caused K^+ loss unequal to Na^+ gain and water loss from tissues. Large intracellular vesicles were found after ouabain treatment. Tissues made Na^+-rich in the cold had approximately one-half as many vesicles as fresh tissues. Rewarming of Na^+-rich tissues, under conditions which Na^+-K^+-ATPase was inhibited, increased the number of vesicles and the tissues lost Na^+ and H_2O. The changes observed on rewarming of Na^+-rich tissues were Ca^{++}-dependent and were not specifically stimulated by Na^+ (K^+, but not Li^+, would substitute for Na^+). Thus, membrane vesicles of rat myometrium have the following characteristics in common with the volume pump: (1) Both are ATP dependent. (2) Both are reduced in the cold and both increase upon rewarming under conditions thought to inhibit Na^+-K^+-ATPase. (3) Both are Ca^{++}-dependent. (4) Neither is specific for Na^+. This evidence supports the hypothesis that membrane vesicles are sites of volume control in rat myometrium.

Pyroantimonate has been widely used as a histochemical tool for the localization of tissue Na^+. Because of its possible usefulness in localizing Na^+-transport sites in myometrium, the pyroantimonate technique was evaluated. Fresh, Na^+-rich and Na^+-poor rat uterine tissues were fixed in OsO_4 with and without added pyroantimonate; then washed and dehydrated as for electron microscopy. At each step both tissues and solutions were analyzed for Na^+, K^+ Ca^{++} and Mg^{++} which precipitate with pyroantimonate. Tissues fixed in the presence of potassium pyroantimonate contained very little of their original Na^+ but retained increased K^+ and much of their Ca^{++} and Mg^{++}, which could account for much of the precipitate observed in tissues. ^{22}Na and ^{124}Sb-pyroantimonate were used to confirm that pyroantimonate does not quantitatively precipitate tissue Na^+. Thus, the pyroantimonate technique is not a valid tool for localizing Na^+-transport sites in the uterus and it should be abandoned for use in other tissues unless they can be shown to behave differently.

In initial studies of rat myometrium fixed by immersion in glutaraldehyde, two populations of cells were observed in the electron microscope. Cells of the minority population were less densely stained and lacked membrane vesicles (termed light cells) as compared with the majority of cells (termed dark cells). A portion of this thesis was designed to define the nature of these two types of cells since, according to the vesicle hypothesis, cells without vesicles should be unable to maintain their volume. It was concluded, after various treatments, that light cells were cells injured during or after removal of the tissues from the animals. The absence of vesicles and density of the light cells could be related to the loss of volume control caused by the injury.

Acknowledgements

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

A. Introduction to Thesis Project

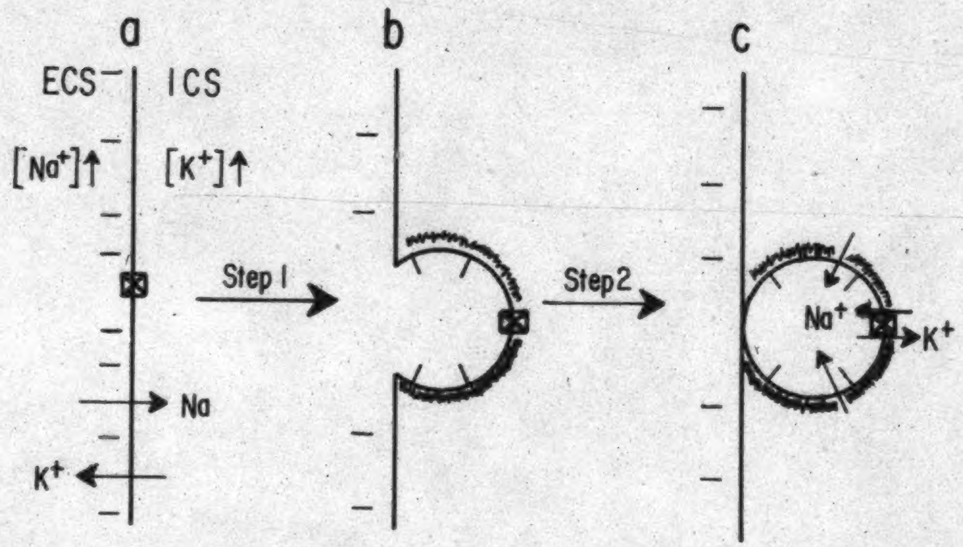
Studies on the distribution of sodium and potassium ions in smooth muscle have indicated that these ions are not simply distributed between cellular and extracellular regions (see Goodford, 1965; Daniel and Robinson, 1970; 1971a,b,c). Among the regions in smooth muscle tissue which could participate in ion compartmentalization are fixed anionic sites on the outer surface of smooth muscle cells (Goodford, 1968; 1970). Goodford *et al.* (1968) postulated that the membrane vesicles of smooth muscle cells could serve as areas of Na^+ accumulation and transport. According to this hypothesis, the flat negatively charged plasma membrane invaginates to form vesicles by an ATP-dependent mechanism as shown in Figure 1. The formation of spherical vesicles brings the charges on the surface closer together and thus increases the charge density within the vesicle. This high concentration of negative charges attracts cytoplasmic Na^+ through the selectively permeable vesicular membrane. Once formed, according to Goodford's calculations, the vesicles are capable of accumulating up to 900 mM cytoplasmic Na^+ based on vesicle size and charge density on the membrane. When the negative charges within the vesicles are balanced by Na^+, the vesicles open to the extracellular space, the charge density decreases and Na^+ escapes accompanied by water movement. The negative charges on the flat membrane are balanced by di-

Figure 1. The vesicular model

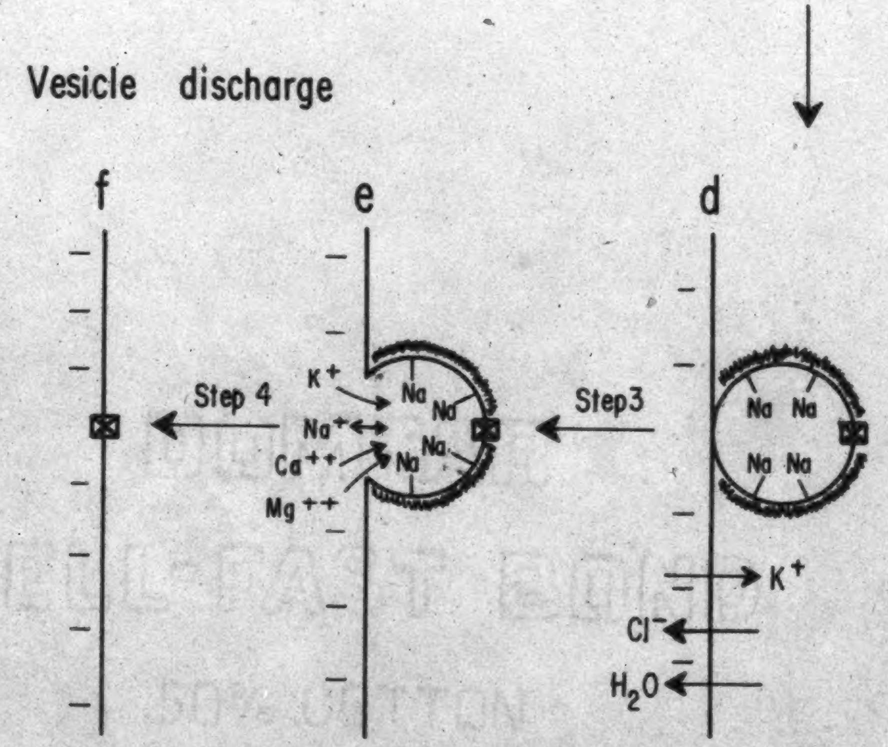
A. Steps 1 and 2 represent the ATP-dependent formation of a spherical vesicle (b and c) from the flat negatively charged membrane (a) which separates the cytoplasm (ICS) from the extracellular space (ECS) and at which the transport ATPase (☒) is located. Once formed (c), the vesicles fill with Na$^+$ by: 1) the ATP-dependent ATPase (Daniel and Robinson, 1971c) and 2) their selective permeability to Na$^+$ (Goodford et al, 1968).

B. Steps 3 and 4 represent the discharge phase. Na$^+$ is bound within the vesicles at the negatively charged sites (d). To maintain electroneutrality Cl$^-$ moves out or potassium moves into the cell (d) after Na$^+$ is bound. Some water follows Na$^+$ into the vesicles and some water moves out of the cell across the flat membrane after binding. The vesicles open (perhaps due to a Ca^{++}-dependent contractile system around the vesicles (〰) Rangachari, 1972) and the bound Na$^+$ exchanges for monovalent and divalent cations in the extracellular space (e). The curved vesicular membrane becomes part of the flat membrane again (f).

A Vesicle formation


a
b
c
ECS
ICS
[Na⁺]↑
[K⁺]↑
Step 1
Step2
Na⁺
K⁺
Na
K⁺

B Vesicle discharge


f
e
d
Step 4
Step3
K⁺
Na⁺
Ca⁺⁺
Mg⁺⁺
Na
Na
Na
Na
Na Na
Na Na
K⁺
Cl⁻
H₂O

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valent cations.

Evidence to support the concept of superficial anionic sites on smooth muscle cells has recently been presented by Goodford and Wolowyk (1972). They found that electron dense, positively charged uranyl ions bound to the external surface of the plasma membrane of glutaraldehyde-fixed tissues. The affinities of cations for the sites were $Ca^{++} = Mg^{++} << K^{+} < Na^{+}$, as determined by the ability of these cations to decrease uranyl precipitate at the cell surface.

Daniel and Robinson (1971a,b,c.) extended the vesicular hypothesis in studies of uterine smooth muscle. They found two exchangeable cellular fractions of ^{22}Na in efflux studies. One fraction of ^{22}Na efflux was found to be relatively fast, the other slow. ^{22}Na in the fast fraction was believed to be ^{22}Na emerging from cells via the membrane vesicles. The slow cytoplasmic fraction was thought to be ^{22}Na leaking out across the flat portion of the plasma membrane. They found that the efflux of ^{22}Na from the fast fraction was reduced but not eliminated by the inhibition of transport ATPase with ouabain. The component of ^{22}Na extrusion which persisted in the presence of ouabain was diminished by ATP depletion. Daniel and Robinson (1971c) postulated that ATP was necessary for : (1) the formation of spherical vesicles and (2) the selective accumulation and binding of Na^{+} within the vesicles as postulated by Goodford (Figure 1). Thus, according to Daniel and Robinson there were two ATP-dependent mechanisms for Na^{+} extrusion both by way of the vesicles: (1) the extrusion of Na^{+} by the ouabain-insensitive, but ATP-dependent, formation of vesicles and binding of cytoplasmic Na^{+} at anionic sites within the vesicles and (2) the extrusion of Na^+ by a ouabain-sensitive transport ATPase associated with the vesicles. Daniel and Robinson (1971c) considered that Na^+ bound within the vesicles exchanged for extracellular cations when the vesicles opened to the exterior.

Evidence that a second (ATP-dependent but ouabain-insensitive) mechanism is involved in Na^+transport comes from the quantitative differences between the effects of metabolic inhibitors and the effects of inhibitors of the transport ATPase (Daniel and Robinson, 1971a,b,c). Accordingly, if only one mechanism were involved both ATP dpeletion and inhibition of the system with ouabain (or K^+-free solutions) should produce the same effects. However, inadequacies in the concept of a single regulatory mechanism were noted as follows (Daniel and Robinson, 1970; 1971 a,b,c): (1) ATP depletion with metabolic inhibitors caused the expected downhill ion movements of Na^+ and K^+ and resulted in swelling of tissues. (2) The inhibition of transport ATPase with ouabain produced downhill ion movements but the tissues did not swell, in fact, they decreased in water content. (3) Analysis of ^{22}Na efflux from uterine tissues showed there were two ATP-dependent systems involved, only one of which was ouabain-sensitive.

In addition to a transport ATPase, a second Na^+-pump has been proposed for other tissues such as red blood cells (Hoffman, 1966), kidney (Kleinzeller and Knotkova, 1964; Whittembury and Fishman, 1969) and skeletal muscle (Leblanc and Erlig, 1969) based on the ability of ouabain to inhibit only part of Na^+ extrusion. The ouabain-insensitive system in kidney was postulated to be an electrogenic Na-Cl pump which controls cell volume (Whittembury, 1968; Whittembury and Fishman, 1969).

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In red blood cells, more recent evidence indicates that the ouabain-insensitive system involves a Na^+-Na^+ exchange process (Smith *et al.*, 1972).

Ethacrynic acid, which was originally thought to selectively inhibit the ouabain-insensitive system in other tissues (Hoffman, 1966; Whittembury, 1968; Leblanc and Erlig, 1969), did not appear to produce volume changes by inhibiting the volume pump directly in smooth muscle but instead inhibited it indirectly by interfering with metabolism (Daniel *et al.*, 1971d). Similar conclusions on the mechanism of action of ethacrynic acid on kidney were reached by MacKnight (1969).

Daniel and Robinson (1971c) showed that whole uterine tissues made Na^+-rich by incubation in a Na^+-rich, K^+-free medium in the cold and then rewarmed in the same solution lost Na^+ accompanied with water by an energy dependent mechanism. Rangachari (1972) and Rangachari *et al.* (1972) confirmed and extended Daniel and Robinson's work on Na^+-rich myometrium. They found that the loss of weight from Na^+-rich tissues into K^+-free solutions was accompanied by a contracture and was inhibited by substances such as isopropylnoradrenaline, papaverine, iodoacetamide (IAAmide) and Ca^{++}-free solutions which prevented contractures of the tissue. Rangachari (1972) proposed that Na^+-rich tissues bound Na^+ within the vesicles in the cold. Upon rewarming, a contractile mechanism, located either around the vesicles or between the vesicles on the flat portion of the membrane, shortened to open and discharge the vesicle contents (Figure 1). Agents which interfered with the contracture were thought to inhibit the contractile process in vesicle discharge.

Similar studies on kidney cells have shown the cells to swell during exposure to the cold and to expel NaCl isotonically during rewarming (Kleinzeller and Knotkova, 1964). A contractile system associated with the membrane of kidney was postulated (Rorive and Kleinzeller, (1972).

The questions asked at the start of this project were: (1) Are the morphological changes which occur in smooth muscle cells in response to inhibitors of metabolism or active sodium transport consistent with the involvement of vesicles in sodium or water transport? (2) If the membrane vesicles are involved in sodium transport and they contain a high sodium concentration, can sodium be demonstrated histochemically with potassium pyroantimonate?

B. Literature Review

1. *Control of Cell Volume*

Cells contain osmotically active materials which are impermeable to the cell membrane as well as existing in an environment of substances which are permeable. This condition leads to the threat of cell swelling and lysis due to the high permeability of water. One mechanism which would keep the water content within cells low would be to pump it out as fast as it enters. However, evidence for the active transport of water has been obtained only for insect cuticle (Beament, 1964), which is not relevant to mammalian cells. Other possible mechanisms which could solve the problem are the evolution of (1) membranes impermeable to sodium, the major extracellular cation; (2) membranes which could withstand large pressure differences; (3) the ability to bind sodium selectively in an osmotically inactive form; (4) a pumping mechanism to transport sodium out of cells as fast as it enters. Plant cells have adopted walls in their evolution which can resist large pressure differences, but at the expense of limited mobility. In contrast, animal cells have evolved mechanisms for ion pumping and/or binding that keep the intracellular concentration of permeable ions, and hence water content, in a steady-state.

Sodium pump concept. An early observation by Macallum (1905) was that potassium of living organisms was confined mainly to the cells; very little was present in the extracellular phase, whereas the opposite was true with respect to sodium. This rule for the distribution of these two cations holds true for nearly all living cells.

The distribution of sodium and potassium has been studied most extensively in three types of tissues *viz.*, muscle, nerve and erythrocytes. Permeability of muscle to potassium was demonstrated by Meigs and Atwood (1916) who found that muscle bathed in KCl solutions would swell and take up potassium. Boyle and Conway (1941) believed that muscle fibers were impermeable to sodium and very permeable to potassium and chloride. However, even at the time of its conception there was mounting evidence against the unmodified Boyle-Conway theory, *i.e.*, Fenn (1938) showed that when potassium was lost from muscle due to fatigue, sodium entered. Furthermore, Heppel (1940) was able to show that injected ^{22}Na would equilibrate with muscle fiber sodium.

Dean (1941) modified the Boyle-Conway theory by introducing the concept of a continuous inward leak of sodium balanced by extrusion of sodium by a pumping mechanism in muscle fibers.

Wilbrandt (1937) referred to the process of ion pumping across cell membranes as active transport. The term active transport was later defined to mean the transfer of a substance across the membrane against its electro-chemical gradient with the expenditure of energy (Ussing, 1949).

The concept that sodium and potassium movements are mediated by a single system comes from several observations: (1) The potassium influx is high in cells which are expelling sodium at a high rate (Maizels, 1951; Ponder, 1950). (2) Sodium is required inside cells and potassium outside for their respective movement (Harris and Maizels, 1952; Glynn, 1957; Whittam, 1962; Sen and Post, 1964). (3) The active extrusion of sodium and uptake of potassium seem to depend on the same metabolic factors (Maizels, 1951).

The steady-state distribution of monovalent cations and water depends upon the leakage and transport of ions into and out of cells (leak-pump hypothesis). Energetic considerations show that any cation crossing the membrane must be accompanied by an anion (usually thought to be Cl^-) or exchange for another cation to maintain electro-neutrality. A transport mechanism which exchanges equal numbers of Na^+ for K^+ is referred to as a neutral pump. An electrogenic pump is a system which extrudes more Na^+ than K^+ are absorbed.

An example of the pump-leak relationship was demonstrated in red blood cells from two strains of sheep by Tosteson and Hoffman

(1960). They showed that ion and water balance were maintained by high pumping activity in cells from one strain of sheep and high K^+ leakage in the other strain.

A neutral pump has been proposed for several tissues such as squid axons (Hodgkin and Keynes, 1955), skeletal muscle (Desmedt, 1953) and smooth muscle (Kao and Nishigama, 1969). However, more recent and complete evidence indicates that the pump is often and perhaps always electrogenic in many tissues including all the above types (see review by Thomas, 1972). Taylor *et al.* (1970) showed conclusively that pumping of Na^+ in Na^+-rich myometrium was electrogenic.

The search for the energy source for active transport led to circumstantial evidence in support of adenosine triphosphate (ATP), a high energy phosphate compound. Sodium efflux in squid axons was markedly inhibited by metabolic inhibitors such as 2,4-dinitrophenol (DNP), cyanide, and azide (Hodgkin and Keynes, 1955). Caldwell (1960a) showed that cyanide treatment resulted in a rapid but reversible decrease in arginine phosphate and ATP in squid axons, and the time course correlated well with decreased sodium efflux. Changes in ATP and sodium efflux were similar when DNP was used to inhibit metabolism. Caldwell (1960b) also injected several high energy phosphate compounds into metabolically inhibited axons and he found that some of them, such as ATP, would increase sodium extrusion. A study of squid axon in which the axonplasm was replaced by salt solutions indicated that 3 Na^+ were extruded for each molecule of ATP hydrolyzed (Baker and Shaw, 1965).

Based on studies with metabolic inhibitors, Maizels and

Whittaker (1940) and Maizels (1951) proposed that ATP was the source of energy for sodium transport by erythrocytes. An accumulation of K^+ occurred when ATP was introduced into erythrocyte ghosts (Gardos, 1954). Hoffman (1960), using red cell ghost preparations loaded with different high energy phosphate compounds, was able to demonstrate that ATP was the primary source of energy for sodium transport.

The dependence of the sodium pump on ATP raised the question whether movements of sodium and potassium might lead to the synthesis of ATP by reversal of the pump. Garrahan and Glynn (1967a) tested this possibility in red cell ghosts. They found that radioactive orthophosphate was incorporated into ATP when the pump was reversed by the entry of sodium and the exit of potassium. The labelling was prevented if ouabain was present.

Schatzman (1953; 1962) demonstrated that the sodium pump was inhibited by cardiac glycosides. The inhibitory effects of these agents were shown to be on the outside of the cell membrane (Whittam, 1958; Caldwell and Keynes, 1959) and these effects could be decreased by increasing potassium concentration externally (Glynn, 1957).

In search of a membrane transporting system, Skou (1957) identified an enzyme in crab nerve which had the following essential properties: The system (1) was located on the cell membrane, (2) had a higher affinity for sodium than for potassium inside the cell membrane, (3) had a higher affinity for potassium than for sodium on the outside of the membrane, (4) contained an enzyme which converted the energy of ATP into the movement of ions, (5) hydrolyzed ATP at a rate dependent upon the sodium concentration inside and potassium outside the cell, (6) was found in cells in which a coupled transport occurred, (7) was inhibited by cardiac glycosides, and (8) displayed the same quantitative relationship of sodium to potassium as the intact cell. The enzyme was therefore termed (Na^+-K^+)-ATPase. The correlation between the properties of this ATPase system described by Skou (1957) and the active transport system for sodium (the sodium pump) have been confirmed and extended (Dunham and Glynn, 1961; Post *et al.*, 1960; Charnock and Post, 1963a; Charnock *et al.*, 1963b; Charnock and Opit, 1968). For example, both systems (1) require ATP; (2) require sodium as well as potassium; (3) have vectorial properties (transport Na^+ out and K^+ in); and (4) are inhibited by low concentrations of ouabain and other cardiac glycosides.

Skou later (1960) postulated, and it has subsequently been shown (Charnock *et al.*, 1963b), that a phosphorylated intermediate was involved in the molecular events of the Na^+-K^+-dependent ATPase in which the hydrolysis of ATP resulted in the transport of sodium and potassium. The exact molecular mechanisms of the sodium-potassium transport enzyme remain unknown; however, several diagrammatic models have been proposed (Opit and Charnock, 1965; Albers *et al.*, 1968; Hokin, 1969).

Other modes of sodium extrusion. Cellular swelling occurs if the sodium pump is inhibited in red blood cells (Tosteson and Hoffman, 1960). However, inadequacies in the concept of only one mechanism controlling ion movements were revealed when inhibition of Na^+ pumping with cardiac glycosides did not completely inhibit Na^+ extrusion in red blood cells (Hoffman and Kregenow, 1966), kidney or muscle (Kleinzeller and Knotkova, 1964; Whittembury, 1968; MacKnight, 1968; Daniel and Robinson, 1971a).

Hoffman (1966) and Hoffman and Kregenow (1966) postulated two sodium pumps for erythrocytes based on the inability of cardiac glycosides to completely inhibit sodium movements. Pump I was composed of two parts both of which were inhibited by cardiac glycosides. Part A was a Na^+-K^+ exchange system. Part B was a sodium-sodium exchange mechanism in which the outflux of sodium required sodium in the external medium. Pump II was defined as glycoside-insensitive but inhibited by ethacrynic acid. ATP was necessary for the operation of pump I. The energy source for pump II was unknown. Garrahan and Glynn (1967a,b) also demonstrated the existence of a second pump in erythrocytes which was similar to pump II described by Hoffman. They also provided evidence for a sodium-sodium exchange mechanism which required ATP but did not result in its hydrolysis. This system was thought to correspond to part B of pump I found by Hoffman.

Evidence for the existence of two sodium extrusion mechanisms has been reported for frog sartorius muscle (Mullins and Frumento, 1963; Horowicz, 1965; Keynes, 1966). One fraction of sodium extrusion was found to be strophanthidin-sensitive and potassium-dependent, whereas the second component was not affected by either. Leblanc and Erlij (1969) found that ethacrynic acid inhibited the second sodium efflux component in frog muscles and thus they proposed that this mechanism was similar to Hoffman's pump II found in red cells.

More recent evidence indicates that ethacrynic acid acts to inhibit glycolysis and oxidative phosphorylation as well as transport

ATPase in a number of tissues (Duggan and Noll, 1966; Jones and Landon, 1967; MacKnight, 1968; MacKnight, 1969; Daniel *et al.*, 1971d). Therefore, the inhibition of Na^+ efflux is thought to be related to ATP depletion by the nonspecific effects of ethacrynic acid. Furthermore, the ouabain-insensitive system in erythrocytes is thought to be a Na^+-Na^+ exchange process independent of the sodium pump (Dunn, 1970; Whittam and Wheeler, 1970; Smith *et al.*, 1972). This exchange system could not lead to volume control. There is also good evidence that the sodium exchange in frog sartorius which persists in the presence of ouabain is an exchange diffusion process (Sjodin and Beauge, 1968; Lubowitz and Whittam, 1969).

Two modes of sodium extrusion, both of which lead to a net sodium exchange are believed to be involved in kidney cell volume control (Kleinzeller and Knotkova, 1964; Whittembury, 1968; MacKnight, 1968; Willis, 1968; Whittembury and Fishman, 1969; Whittembury and Proverbio, 1970). These investigators have found that kidney cells can extrude sodium with chloride and water even in the presence of ouabain which inhibits the exchange of sodium for potassium. Furthermore, when tissues were loaded with sodium by immersion in cold medium without potassium and then rewarmed in a medium without potassium, a net sodium extrusion from cells accompanied by efflux of chloride and by volume loss but without potassium uptake was noted. If potassium was added to the rewarming solution, a gain of potassium occurred reciprocal to the loss of sodium. This exchange was inhibited by ouabain, but ouabain did not interfere with the extrusion of sodium with chloride according to the above investigators. Ethacrynic acid was observed to inhibit extrusion of sodium with chloride and water but it had little or no effect on the exchange of sodium for potassium (Whittembury, 1968; Whittembury and Proverbio, 1970). MacKnight (1968), however, showed ethacrynic acid to be a metabolic poison.

Cell volume in kidney cells could be maintained by (1) water movement associated with sodium transport coupled either directly or indirectly to potassium; (2) water movement associated with the transport of sodium and chloride without any link to potassium entry. The quantity of water the first system could transport would depend upon the coupling stoichiometry and leak rates of the ions involved. The second, ouabain-insensitive, system could move large quantities of water and it has the following characteristics (Kleinzeller and Knotkova, 1964; Whittembury and Proverbio, 1970; Rorive *et al.*, 1972; Rorive and Kleinzeller, 1972): (1) The solution extruded from cells consists of 140 mM NaCl (essentially isotonic). (2) The extrusion system is dependent upon metabolic energy. (3) The mechanism is not specific for sodium, substances such as lithium, potassium, choline, or Tris [tris(hydroxymethyl)aminomethane] can substitute for sodium. (4) The system does not require potassium in the external solution. (5) The ouabain-insensitive mechanism is pH dependent and it requires calcium in the external solution.

Willis (1968) proposed that a pump located within a restricted space between kidney tubular cells or within folding of the membrane (a crypt pump), inaccessible to ouabain action, could account for all the properties of the ouabain-insensitive pump. The crypt pump hypothesis is questioned on the grounds that it is difficult to see why ouabain should not have access to the sites of the Na$^+$ pump if ouabain clearly affects K$^+$ transport (Whittembury and Proverbio, 1970). Furthermore, many cells in which such a system exists do not have membrane crypts, *i.e.*, red blood cells, ascites tumor cells or smooth muscle cells. Also this model does not explain the extrusion of choline$^+$ and Tris$^+$ which are not transported by membrane ATPase but which do substitute for Na$^+$ in the ouabain-insensitive pump (Rorive and Kleinzeller, 1972).

Rorive and coworkers (1972) and Rorive and Kleinzeller (1972) have proposed that intracellular water and electrolytes are extruded by a ouabain-insensitive mechanochemical process in kidney cells. This mechanochemical system is proposed to operate by a Ca^{++}-activated ATPase which initiates a membrane contractile mechanism to extrude isotonic solution from cells. The lack of substrate specificity and ouabain insensitivity are consistent with a mechanochemical system.

The mechanochemical hypothesis for volume control involves the existence of a contractile mechanism. Contractile mechanisms responsible for water movement have been described for a number of cells, *e.g.*, the contractile vacuole of protozoa (Organ *et. al.*, 1968; Perkins and Jahn, 1970) and tumor cells (Hoffman-Berling, 1956). Contractile systems are dependent upon contractile proteins. Contractile proteins associated with membranes have been reported in erythrocytes (Marchesi *et. al.*, 1969), liver cells (Neifakh *et al.*,

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1965) and thrombocytes (Bettex-Golland and Luscher, 1965). The existence of actomyosin in mitochondrial membranes (Vignais *et al.*, 1963; Neifakh *et al.*, 1965) and the demonstration that ion movements occur during ATP synthesis by oxidative phosphorylation (Green, 1970) support a mechanochemical system.

Daniel and coworkers (1962) were unable to show any significant effects of potassium removal on the rate of ^{22}Na loss from rat and rabbit uterine smooth muscle. Therefore, Daniel (1963b) proposed that the Na^+ and K^+ pumps were not coupled and that the sodium pump operated at a faster rate than that of the potassium pump, giving it an electrogenic nature. Further support for the electrogenic nature of the sodium pump in smooth muscle has been presented (Burnstock, 1958; Casteels, 1969; Daniel *et al.*, 1969; and Taylor *et al.*, 1970).

Evidence for two mechanisms regulating ion distribution in uterine smooth muscle has been presented by Daniel and Robinson (1970, 1971a,b,c). They proposed, on the basis of ^{22}Na efflux studies, that sodium exits from four compartments in this tissue (termed A to D). They found compartment A was large and contained quantitatively about that amount of sodium predicted from the size of the extracellular space. Sodium in compartments B and C was viewed as emerging from cells via a metabolically dependent transport mechanism. Compartment D was described as a small bound fraction which exchanged only very slowly with ^{22}Na. Metabolic inhibition, which depleted uterine tissues of adenine nucleotides, resulted in decreased ^{22}Na efflux from both cellular fractions (B and C) and resulted in tissue swelling. Potassium-free solutions and ouabain had only slight effects on sodium-rich tissues. Ouabain decreased efflux of sodium from the fast cellular fraction B.

Daniel and Robinson noted that in both ouabain and potassium-free solutions the water content of tissues decreased and both inhibitors failed to interfere with the isotonic extrusion of water when cold, sodium-rich tissues were rewarmed. A ouabain-insensitive, ATP-dependent system was therefore postulated for control of cell volume in addition to the classical ouabain-sensitive ATPase.

Daniel and Robinson proposed (1971c) that membrane vesicles of smooth muscle cells might be sites of sodium transport (see introduction pages 1 to 5). The properties of this vesicular mechanism, which is thought to control cell volume (Daniel and Robinson, 1970; 1971a,b,c; Rangachari, 1972; Rangachari *et al.*, 1972), are similar to the mechanochemical system of kidney cells (Rorive *et al.*, 1972; Rorive and Kleinzeller, 1972). Both systems (1) require metabolic energy; (2) lead to the isotonic extrusion of NaCl from Na^+-rich tissue; (3) are not specific for Na^+; (4) do not require external K^+ and are not inhibited by ouabain; and (5) may involve a mechanochemical process.

Membrane invagination or vesiculation may be a required step in the activation of some membranes or enzymes for the movement of molecules across them. Green (1970) has discussed the conformational basis of energy transductions in biological systems. He suggested that changes in mitochondrial membrane configuration provide the bond energy necessary for ATP synthesis and production of ion gradients. Conversely, ATP hydrolysis could lead to comformational changes in membrane with movements released by the ATPase reaction, inhibits the activity of tissue phosphatases (Rosenthal *et al.*, 1969). The same workers also demonstrated that lead catalyzes the non-enzymatic hydrolysis of ATP used as the substrate for release of phosphate.

Membrane vesicles are a characteristic feature of all animal cells and some plant cells (Rustad, 1959). The membrane vesicles have been termed caveolae intracellulares in smooth muscle (Caesar *et al.*, 1957; Gabella, 1971) and plasmalemma vesicles (Bruns and Palade, 1968a, b) or micropinocytotic vesicles in endothelial cells (Bennett, 1956).

The term caveolae intracellulares may be unsuitable since the vesicles have never been shown to be *intra*cellular. The term pinocytotic is also improper, because vesicles in smooth muscle have never been shown to be involved in any uptake process. The term plasmalemma vesicles is not usually applied to smooth muscle. Therefore, in this thesis, the vesicles observed in smooth muscle will be referred to as *membrane vesicles* since their origin is believed to be the plasma membrane and this term seems more appropriate.

Association-induction hypothesis. The usual approach to the concept of volume control in cells is based on the membrane theory. According to this theory, the cell membrane is the limiting barrier to solutes between the cell interior and the external environment. A sodium pump located on the membrane (as described above) is responsible for maintaining the steady-state of ions and water. Another assumption of the membrane theory is that ions and water are relatively free within the cell.

of water and ions. In search of support for a configurational model, Penniston and Green (1968) found ouabain-insensitive conformation changes in the form of pinocytotic vesicles in red cell ghosts in response to ATP. From this, their general conclusion was that conformational changes such as pinocytosis could be necessary processes in the movement of ions across membranes.

An interesting theory of ATP production by mitochondrial membranes has recently been submitted by Banks and Vernon (1970). They postulate that the process may operate as follows: (1) The membrane contains an ATPase but it normally is held in an inactive, extended configuration. A molecule of ADP and inorganic phosphate become attached to the enzyme. (2) The membrane now takes up a folded configuration thereby extruding water and generating a non-aqueous microenvironment. (3) A metal cation enters the folded configuration perhaps accompanied by water and activates the ATPase which in turn catalyzes the formation of ATP. (4) The activating cation passes out of the folded configuration and the membrane takes up the original extended configuration with the release of ATP. It is not difficult to see how this mechanism could result in ion transport if it operated in reverse order and if the process of folding and extension released an ion at a site different from the origin of the ion.

Additional evidence suggesting vesicular involvement in sodium transport in smooth muscle was the demonstration of high ATPase activity as demonstrated histochemically in membrane vesicles (Rostgaard and Barrnett, 1964; Lane, 1967). However, the validity of this histochemical technique for localization of ATPase activity has been questioned on the grounds that the lead cation used to capture phosphate

An alternative theory based on the interaction of electrolytes with selective binding properties of proteins was formulated by Ling (1962) and termed the association-induction hypothesis. According to this view, intracellular solutes exist in two states: (1) in solution in cell water, and (2) adsorbed onto cell proteins. ATP is thought to energize cell proteins to maintain a particular cooperative state necessary for selective ion binding. Thus, as ATP is adsorbed onto a controlling site on a protein, it influences a fixed number of anionic sites to selectively adsorb K^+. Without ATP, other cations such as Na^+ may occupy the anionic sites. Ouabain is thought to shift the affinity of the system from K^+ to Na^+ (Ling, 1969). Cell water is thought to exist in a physical state different from that in the extracellular solution (Ling, 1966). It is pictured as existing in polarized multilayers about the protein. Cell volume equilibrium depends upon the reversible forming and breaking of salt linkages in the protein matrix and the association of free counter ions such as K^+.

Certain evidence supports the association-induction hypothesis. Cope (1967) suggested that approximately 70% of cell sodium was in a complexed state and therefore did not contribute to the nuclear magnetic resonance (NMR) spectrum. NMR studies of water have also indicated structuring of this component (Hazelwood *et al.*, 1969). Hinke and McLoughlin (1967) provided further support for the complexing of intracellular sodium and potassium in barnacle muscle studied with cation-sensitive microelectrodes. Recent spin-echo studies substantiate the idea that ordered water exists in biological tissues (Cope, 1970).

Most evidence, however, is in favor of the membrane theory as summarized below: (1) Living cells are approximately isotonic with 0.9% NaCl solutions, which demands that the osmotic activity within cells be equal to that of this solution. To achieve this, nearly all the ions within cells must be in a free state. (2) The cytoplasmic resistance is relatively low as compared with high membrane resistance. The low cytoplasmic resistance is interpreted as indicating nearly complete dissociation of intracellular ions. (3) The mobility of K^+ in cells is equal to that of free K^+ in solution (Hodgkin and Keynes, 1955). (4) The correct magnitude of the resting and action potentials can be predicted from the Nernst equation (Hodgkin and Katz, 1949) which is based on complete dissociation of intracellular K^+. (5) The transport of ions across frog skin separating two ion bathing solutions (Ussing, 1954) could not be accounted for on the basis of ion binding. (6) A linear relationship between potassium content and ATP concentration does not exist in smooth muscle as predicted by the association-induction hypothesis (Rangachari *et al.*, 1972).

2. *Localization of Sodium with Potassium Pyroantimonate*

Use of potassium pyroantimonate. Sodium transport sites in smooth muscle cells could be demonstrated histochemically if sodium were concentrated at the transport sites and if a valid histochemical tool were available for sodium localization. Potassium pyroantimonate has been widely used as a tool to localize sites of high sodium concentration in tissues.

Komnick (1962) and Komnick and Komnick (1963) proposed that potassium pyroantimonate could be used to localize sodium in tissue prepared for the electron microscope. This claim has been recently reaffirmed (Komnick and Bierther, 1969; Lennep and Komnick, 1971). Potassium pyroantimonate ($K_2H_2Sb_2O_7.4H_2O$) is added to the fixative and the pyroantimonate anion is thought to form insoluble, electron dense precipitates with sodium at sites of high sodium concentration in tissue.

Kidney tissue has been studied by this method in an effort to localize areas of high sodium content or sites of sodium transport. Amakawa and Mizuhira (1968) and Nolte (1966) found precipitates localized in the brush border of proximal tubule cells of rat kidney. Precipitates have also been observed along the basement membrane in distal tubules and in glomeruli (Tandler and Kierszenbaum, 1971). Nuclear precipitates found in kidney preparations were thought to represent bound sodium, while precipitates found associated with the membrane were thought to demonstrate sodium transported by the membrane (Tisher *et al.*, 1969). Based on precipitate location, an osmoregulatory function for chloride cells of the kidney of the stickleback has been proposed (Komnick and Bierther, 1969).

Brain and nervous tissue have been investigated and pyroantimonate precipitates were thought to demonstrate mainly bound sodium (Hartmann, 1966; Villigas, 1968; Siegesmund and Edelhauser, 1968). Increased precipitates were found in vesicular structures of astrocytes following intracisternal injection of sodium chloride solutions (Torack, 1971).

Pyroantimonate precipitation on the external surface of the stratum cornea cells of frog epidermis was interpreted as support for a standing-gradient osmotic flow process for this tissue (Henrikson, 1970; Lennep and Komnick, 1971). Sodium was thought to be mobilized from frog epithelial nuclei and rat kidney nuclei during stimulation of active transport by vasopressin. These observations were based on pyroantimonate location and ^{22}Na movement in fresh isolated nuclei (Zadunaisky *et al.*, 1968).

Other tissues have been investigated using pyroantimonate to localize sodium. These tissues include the spiral organ of the ear (Vinnikow and Koichev, 1969), plant cells such as maize roots, bean embryo and spinach chloroplasts (Nobel and Murakami, 1967; Tandler *et al.*, 1970), sweat glands (Ochi, 1968), salt excreting glands (Lennep, 1968), testis (Kierszenbaum *et al.*, 1971), vas deferens (Lane and Martin, 1969), liver (Tandler *et al.*, 1970), bone marrow and blood cells (Spicer *et al.*, 1968; Hardin *et al.*, 1969; Spicer *et al.*, 1969; Hardin and Spicer, 1970; Clark and Ackerman, 1971a,b).

Heart muscle has been studied after fixation with pyroantimonate (Shinna *et al.*, 1969, 1970; Legato and Langer, 1969; Klein *et al.*, 1970; Thureston-Klein and Klein, 1972) as has skeletal muscle (Zadunaisky, 1966; Shiina and Mizuhira, 1970). No comparable studies have been reported for smooth muscle.

Validity of pyroantimonate method. Evidence that pyroantimonate precipitates contain sodium in tissue fixed for electron microscopy comes from various sources. The presence of sodium in tissue precipitates has been demonstrated by electron probe analysis (Lane and Martin, 1969; Tandler *et al.*, 1970; Tandler and Kierszenbaum, 1971). Selected area diffraction pattern studies have shown that precipitates contained sodium (Hartmann, 1966). Studies of the combined use of pyroantimonate and ^{22}Na autoradiography have shown an association between the developed grains of Na^+ and precipitates (Amakawa *et al.*, 1968; Shiina *et al.*, 1969; Tisher *et al.*, 1969). Others have reported that tissue precipitates were decreased or increased under conditions which lower or raise the tissue sodium content or increase or decrease sodium extrusion (Kaye and Donn, 1965; Hartmann, 1966; Zadunaisky *et al.*, 1968; Tani *et al.*, 1969; Torack, 1969; Shiina and Mizuhira, 1970; Satir and Gilula, 1970). A study on the efflux of ^{22}Na from ^{22}Na-loaded tissue during fixation indicated that slightly more ^{22}Na remained in tissue when pyroantimonate was present (Zadunaisky, 1966).

There are, however, several reasons for doubting the validity of this technique: (1) Pyroantimonate solutions precipitate *in vitro* with solutions of other cations and cellular substances such as calcium, magnesium, zinc and biogenic amines (Bulger, 1969; Lane and Martin, 1969; Shiina *et al.*, 1970) and evidence has been presented to show that pyroantimonate precipitates with glycogen and histones in tissues (Clark and Ackerman, 1971b). (2) Tissue precipitates were found to contain calcium, magnesium and potassium as well as sodium and antimony as demonstrated by electron probe analysis (Tandler *et al.*, 1970; Kierszenbaum *et al.*, 1971; Tisher *et al.*, 1972). (3) Potassium pyroantimonate has been shown to be insoluble in dehydrating solutions used to prepare specimens for study (Tice, personal communication; Shiina *et al.*, 1970; Tisher *et al.*, 1972). (4) Precipitates were often not found in some cells when glutaraldehyde was used as the fixative and the pattern of precipitate varied with the fixative used (Lee *et al.*, 1967; Bulger, 1969; Clark and Ackerman, 1971a; Sumi, 1971; Sumi and Swanson, 1971). In addition, there is the possibility of diffusion artifacts during fixation and dehydration.

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Klein and Thureston-Klein (1972) used flame photometry to analyze precipitates formed *in vitro* with sodium and pyroantimonate solutions. They found a linear relationship between sodium-added and precipitate formed. However, isolated nuclei, spleen and sodium impregnated gels analyzed by a similar flame photometric method and nuclear activation analysis after pyroantimonate-osmium tetroxide fixation showed that the gels and tissues contained little sodium and increased contents of potassium (Spicer *et al.*, 1972).

3. *Light and Dark Cells*

Significance of this study. During initial examination of uterine smooth muscle in the electron microscope, two populations of cells were found in the same tissue preparation. Cells of the minority population were less electron dense and randomly distributed among the more dense cells. Other distinguishing characteristics, such as lack of membrane vesicles and swollen endoplasmic reticulum and nuclear envelope, were noted between the two types of cells. Similar observations with light and electron microscopy of smooth muscle and other tissues have implicated contraction-relaxation or the method of fixation as the factors responsible for density differences (see Aita *et al.*, 1968a; Dohrman, 1970). Since cells without vesicles should be unable to maintain their volume (Chapter 2), a portion of this project was designed to study the differences between the two types of smooth muscle cells and to try to account for them.

Light and dark smooth muscle cells. Two types of smooth muscle cells differing in staining densities were first distinguished in 1844 by Kolliker (Quoted by Conti *et al.*, 1964). Later Henneberg in 1901 studied carotid artery smooth muscle of pig, beef and humans in contracted and relaxed states. He found two types of cells which he termed clear and dark cells. Clear cells had a round or elliptical cross-sectional shape, a clear cytoplasm and a thick nucleus. The clear cells were thought to characterize contracted muscle. Dark cells had a prismatic shape and an intensely colored cytoplasm and were seen in relaxed smooth muscle (quoted by McGill, 1909; and Aita *et al.*, 1968a). This view was immediately opposed by Heidrich in 1902. He studied smooth muscle of the intestine, urogenital system and blood vessels from a number of animals and concluded that dark cells were contracted cells (quoted by McGill, 1909). The structure of the same smooth muscle from different animals was reviewed by McGill (1909). She described the syncytial nature of smooth muscle and studied the structure of contracted and relaxed cells. She found contracted cells to be shorter, wider and more densely colored than relaxed cells.

The concept that dark smooth muscle cells as viewed through the light microscope were cells contracted at the time of fixation was generally accepted. The only opposing report was that made earlier by Henneberg in 1901. However, Hirsch (1955) believed like Henneberg that vascular smooth muscle light cells were relaxed.

Guinea pig, rat and human intestinal and rat uterine smooth muscle were studied in the phase contrast and electron microscope by Gansler (1961). She described dark cells as having a prickly appearance due to the narrow pointed cytoplasmic projections in contrast to the rounded structure of light cells with wide, blunt extensions. Light cells were found to have a light ground substance with disorganized filament structure, whereas dark cells had a very dense cytoplasm with thin filaments of no consistent pattern. Light cell nuclei were less dense than those of dark cells. The dark cell membranes were difficult to see against the dense myoplasms but contained numerous membrane vesicles. Membrane vesicles were often absent from the light cells. The difference in density of the two types of cells was thought to be dependent upon contraction as dark cells were believed to be more numerous in tissue contracted at the time of fixation. Contraction was thought to lead to cell shrinking by loss of intracellular water. The result of water loss was thought to produce a condensation of the myofilaments making the cells more dense when viewed under the microscope.

Laquens and Lagrutta (1964) reported finding light and dark smooth muscle cells in human uterine tissues during pregnancy. Jaeger (1964) referred to two types of smooth muscle cells found in the human pregnant and nonpregnant myometrium as "resting" and "active". The resting cells were less dense than those termed active. He concluded that the cells were in various states of contraction at the time of examination.

Histological and histochemical studies indicated that light and dark cells of vascular smooth muscle were not due to contraction or relaxation (Conti *et al.*, 1964; Aita *et al.*, 1968b). Light and dark cells were believed to be two different types of cells from a morphological and functional point of view. These authors have referred to these cells as round or oval for light cells and triangular or star-shaped for dark cells, after their cross-sectional appearance. However, more recently they have discarded their original claims and returned to the contraction-relaxation hypothesis (Conti *et al.*, 1972). They found in arteries relaxed prior to fixation some light cells which they believed were contracted cells. In contracted arteries, they found some dark cells which were described as relaxed cells. They concluded that the density of a cell was dependent upon the contracted or relaxed state of the cell at the moment of fixation. However, they viewed the contracted cells as the light cells which is opposite to the conclusions of Gansler (1961).

The nature of density differences observed in stretched mammalian and turtle oviduct smooth muscle has recently been claimed to reflect an unusual osmotic response of occasional cells to the glutaraldehyde-buffer combination (Somlyo *et al.*, 1971a,b). They proposed that the electron density of smooth muscle cells was due to the degree of hydration at the time of fixation, since prior incubation of tissues in hypertonic solutions increased the density of cells.

Light and dark cells of other tissues. An abundance of literature has accumulated over the past 80 years on the nature of liver and brain cells which vary in density when viewed with the light and electron microscope. Early reports characterized dark liver cells as those involved in bile production and much work was devoted to their behavior toward specific dyes. Unequal staining of brain and spinal ganglion cells were seen and dark cells were believed to be sympathetic cells (see review Scharrer, 1938).

The appearance of light and dark liver and brain cells could be avoided by intravascular perfusion fixation. Therefore, Scharrer (1938) thought that dark and light cells might be brought about artificially by pressing or tearing of fresh tissues when they were removed for immersion fixation. He proposed that, as a consequence of mechanical injury, a breakdown in the colloidal system of the cell occurred with instantaneous loss or inhibition of water, resulting in dark stained shrunken cells or light swollen cells.

More recent studies indicated that dark cells increased in number after various toxic treatments to rats (Herdson *et al.*, 1964; La Fontaine and Allard, 1964; Wood, 1965; Allen and Carstens, 1966), mice (Papadimitriou, 1965), monkeys (Ghidoni, 1967) and rabbits (Witzleben, 1966). However, dark cells were regarded as a reaction due to injury after removal of tissues, when it was observed that dark cells also increased in number in control tissues not subjected to poisonous substances but also fixed by immersion (Steiner and Bablio, 1963; Theron, 1965).

Ganote and Moses (1968) noted that conditions which tended to result in inadequate fixation of liver tissue increased the frequency of both light and dark cells and intermediate density cells on the surface of tissue blocks. Dark cells were found more frequently in the central portion of tissue blocks. After *in situ* fixation, intermediate density cells were observed and therefore, light and dark cells were thought to be artifacts of fixation. This conclusion was similar to that made earlier by Scharrer (1938) who considered light and dark cells as artifacts due to mechanical injury prior to fixation.

Light and dark epithelial cells of choroid plexus of the brain from dog, mouse and humans were considered to be due to varying states of cell hydration at the time of fixation (Dohrmann, 1970). They were not believed to be artifacts, since they could be demonstrated in tissue fixed either by immersion or perfusion.

CHAPTER 2

AN ULTRASTRUCTURAL STUDY OF THE CONCEPT OF MEMBRANE VESICULATION AS A MECHANISM FOR ACTIVE TRANSPORT

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CHAPTER 2

AN ULTRASTRUCTURAL STUDY OF VESICULAR TRANSPORT IN RAT MYOMETRIUM

A. Objectives

If membrane vesicles of smooth muscle are involved in Na^+ and water transport in the way postulated (see introduction), several changes should occur in vesicular structure and electrolyte content following treatment of tissues with metabolism or transport inhibiting substances.

1. If ATP is necessary for membrane vesicle formation and maintenance, depletion of ATP with metabolic inhibitors should eliminate vesicle formation and lead to their destruction.
2. If membrane vesicles are sites of sodium transport, [ATP] reduction with metabolic inhibitors should precede or accompany vesicular changes, Na^+-gain, K^+-loss and volume changes.
3. If the ouabain-insensitive volume pump is the only mechanism associated with the vesicles, inhibition of transport ATPase with ouabain should not directly affect vesicular structure.
4. If vesicle formation and discharge involve a Ca^{++}-dependent mechanochemical system as proposed by Rangachari (1972), rewarmed Na^+-rich tissues should have altered vesicle structure or number than those noted in cold Na^+-

tissues. This alteration in vesicle size or in number could be prevented by substances such as Ca^{++}-free solutions and iodoacetamide which interfere with contractility.

The objective of this study was to test the vesicular hypothesis by evaluating the above predictions. Some pieces of myometrium were analyzed for cation contents and ATP and other pieces of tissue were prepared and examined in the electron microscope after incubation in Krebs-Ringer solution with and without metabolism (IAA ± DNP) and transport (ouabain, 5°C) inhibitors.

B. Materials and Methods

1. *Tissue Preparation*

Female Wistar rats weighing approximately 100 grams were injected subcutaneously with 50 micrograms of diethylstilbesterol daily for six days. Each rat was killed by a blow to the head. The abdominal cavity was cut open and the uterus rapidly removed. The two uterine horns were separated at the bifurcation and each horn was spread out on filter paper dampened with Krebs-Ringer solution. In all tissues used in this study, the endometrium was separated from the myometrium by stripping the two layers apart. The endometrial portion, which also contained much of the circular muscle, was discarded.

All myometrial portions were suspended on stainless steel hooks. The tissues were allowed to recover from handling for one hour in Krebs-Ringer medium at 25°C aerated with 95% O_2-5% CO_2.

Recovered tissue was designated as *fresh tissue*. Fresh tissues were weighed and transferred to separate tubes for their respective treatments. Tissues were made *sodium-rich* by incubating them overnight at 5°C in nonaerated potassium-free Krebs-Ringer solution.

After treatment, a piece of tissue approximately 5 x 5 x 0.5 mm was removed from the center of each uterine horn for electron microscopy. The two remaining pieces were placed together for ATP or cation analysis.

In measurements of fresh tissue, tissue from one uterine horn was used as the control and compared with its paired, treated horn. In measurements of sodium-rich tissue, comparisons were made between tissues removed from the same uterine segment before and after rewarming.

2. *Solutions*

The composition of Krebs-Ringer solutions used in this study is shown in Table 1.

Unless otherwise specified, chemicals and drugs used in this study were added directly to the Krebs-Ringer incubation solution. The sources for the chemicals used are given in the appendix.

3. *ATP Determination*

The ATP contents of tissues were estimated by the firefly method (Kahlen and Koch, 1967; Wirth *et al.*, 1970).

TABLE 1

Composition of solutions

Salts	Normal Krebs-Ringer (mmoles/liter)	K^+-free Krebs-Ringer (mmoles/liter)	K^+- Krebs-Ringer (mmoles/liter)	Li^+- Krebs-Ringer (mmoles/liter)
NaCl	115.0	120.0	-	-
$NaHCO_3$	21.9	21.9	-	-
KCl	4.63	-	120.0	-
LiCl	-	-	-	138.0
$CaCl_2$	2.47	2.47	2.47	2.47
$MgSO_4$	1.16	1.16	1.16	1.16
$LiCO_3$	-	-	-	16.0
Glucose	49.0	-	-	-
$KHCO_3$	-	-	21.9	-
Sucrose	-	49.2	49.2	49.2

ATP was extracted by placing the tissues for 10 minutes in beakers containing 10 ml of boiling glycine buffer (2.0 mM; pH 9.5-10). Thereafter the solutions were transferred to graduated test tubes and the volumes readjusted to 10 ml. The test tubes were placed on ice until ready for estimation.

Soluble extract (luciferin-luciferase) from 50 mg of dried firefly lanterns (Sigma) were ground with a mortar and pestle and dissolved in 400 ml of cold 0.1 M arsenate buffer (pH 7.4). $MgSO_4$ (to make 0.02 M) was added to the enzyme solution prior to use. 5 ml of the enzyme solution was placed in scintillation vials and stored in the cold until used.

ATP standards (0.05 - 1.0 µg/ml) were prepared from frozen stock solutions (1 mg ATP/ml glycine buffer) at the same time that the ATP was extracted from tissues.

0.5 ml of the standards or tissue extracts was added to 5 ml of the luciferin-luciferase solution and mixed by swirling the vial. 30 seconds after mixing the two solutions, each vial was counted three consecutive times for 0.1 minute. A liquid scintillation counter (Nuclear-Chicago) set up for integral counting on one wide open channel was used in this study.

The amount of ATP in the tissue extracts was estimated by comparison with a standard curve (drawn on log-log paper) of the ATP concentrations in the standard solutions against the average counts produced in the enzyme-ATP reaction.

4. *Determinations of Ion Contents of Tissues*

Tissues were placed in dried, weighed test tubes and the *wet weights* obtained. The tissues were dried to a constant weight *(dry weight)* at 105°C. The total tissue water content was calculated as the difference between wet and dry weights (g H_2O/g solids). Concentrated nitric acid and 30% hydrogen peroxide (0.1 ml each) were added to each dried sample and then the samples were digested on a hot sand bath. Blank test tubes were treated in a similar manner. The resulting residues were dissolved in de-ionized distilled water and the volumes made up to 25 ml. Sodium and potassium contents were determined using an EEL flame photometer. A standard curve for each experiment was constructed from standard solutions of sodium or potassium chloride. Ionic contents of tissues were estimated by comparison of sample readings with the standard curves after subtracting the blank readings.

5. *Electron Microscopy*

Preparation. Tissues were fixed by immersion in about 5 ml of 5% glutaraldehyde, buffered with Millonig's phosphate buffer (pH, 7.4; 960 mOsm) at 25°C. The pieces of fixed tissue were stored in the cold until ready for postfixation and dehydration. The tissues were then washed three or four times in phosphate buffer, and postfixed in 1% osmium tetroxide with Millonig's buffer for one hour. After rinsing twice in distilled water, samples were dehydrated in a series of graded concentrations of alcohols followed by propylene oxide.

All tissues were embedded in Epon 812 in flat molds oriented so the outer longitudinal muscle layers would be cut in cross-section. Pyramids with face dimensions less than 0.5 x 0.5 mm were cut with a razor blade on the serosal surface of the tissue over the longitudinal muscle layer. Silver to grey sections were cut using a diamond knife with a Porter-Blum ultramicrotome (MT2). All sections were placed on bare 300 mesh copper grids and examined in a JEM-7A electron microscope after double staining with uranyl acetate (Gibbons and Gristone, 1960), and lead citrate (Reynolds, 1963).

Quantitative measurements. The following procedure was followed to assure random sampling in quantitating the membrane vesicles in smooth muscle cells.

A total of 16 photographs were taken at each time after the various treatments (Tables 2 - 12; 4 photos/tissue x 4 tissues/time).

One photograph (x 8,000 magnification) was taken of sections overlying the center square (or closest square containing a section) from each quadrant of each grid (4 photographs per grid). The areas photographed (ca. 1/16 of the grid square; ca. 1/300 of the section area) had to contain at least 50% cells which were cut in cross section. If this criterion was not met in the lower right area of the grid square, the lower left, the upper left, and then the upper right areas were considered for photography until a suitable area was found. If sections contained sectioning or staining artifacts, they were rejected and the tissues resectioned and stained.

Negatives were enlarged 3 times (x 24,000 magnification) and printed on 8" x 11" paper. A map tracing compass was used to measure distances along smooth muscle cell membranes in the photographs. All surface vesicles (within 3 mm of the surface) of the cells in the photographs (ca. 10 cells/photo) were counted. From the measurements taken from photographs, the number of vesicles per millimeter of membrane for each tissue was estimated.

The center portion of each negative was enlarged 10 times (x 80,000 magnification) (if the center was unsuitable, the left center then right center areas were considered). A compass and millimeter scale were used to determine the average size of each vesicle in the enlarged field. Two measurements (one parallel and one perpendicular to the field) were made of each vesicle at the maximum diameter. From the above data, the average size of the vesicles in the field (ca. 20 vesicles/field), the vesicular volume and vesicular surface area were calculated. The total volume and total surface area per unit length of membrane were obtained by multiplying the average number of vesicles/mm membrane for each photograph by their average volume or surface area.

The average vesicular number per unit length and dimensions of each tissue were determined from the 4 photographs taken of each tissue. These data were compared with corresponding dimensions determined for the paired horn.

6. *Statistics*

The paired student's t-test was used to test for statistical significance between values obtained from paired tissues. A "p" value of < 0.05 was chosen as the level of significant differences between tissues.

C. Results

1. *Appearance of Control Tissues*

Figure 2 shows a cross-sectional view of the longitudinal layer of the rat myometrium. The smooth muscle cells of the myometrium are irregular in shape with occasional projections. Membrane bound vesicles appear intermittently along the plasma membrane of the cells. A vesicle at high magnification is seen in Figure 3. Both the flat membrane and vesicular membrane are well defined, and have a typical tripartite layered structure approximately 80 Å thick. Many vesicles appear open to the extracellular space as shown in Figures 2 and 3. However, some membrane vesicles can be seen which have no apparent communication to the exterior of the cell (Figure 2). This could be because the connections were outside the plane of section or because the vesicles were free within the cell.

To test whether there were any closed vesicles, tissues were fixed in 5% glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4, Revel and Karnovsky, 1967) and containing colloidal 1% lanthanum nitrate. Postfixation was in 1% osmium tetroxide solution with the same buffer. These tissues were examined unstained in the electron microscope.

Figure 4 shows that the colloidal suspension of lanthanum nitrate penetrated between cells. Lanthanum was present in vesicles open to the extracellular space and also in vesicles with no apparent opening to the outside.

Particles (~100 Å) associated with the inner surface of the vesicular membrane were outlined by the dense lanthanum deposit (Fig-

Figure 2. Smooth muscle cells of the longitudinal muscle layer of the rat uterus. Membrane vesicles appear along the surface of the cells. Single arrows indicate vesicles open to the extracellular space. Vesicles with no apparent openings are shown with double arrows. Magnification x24,000.

Figure 3. A high magnification of a membrane vesicle. Note the triple layered membrane, the opening in the extracellular space. Magnification x213,000.

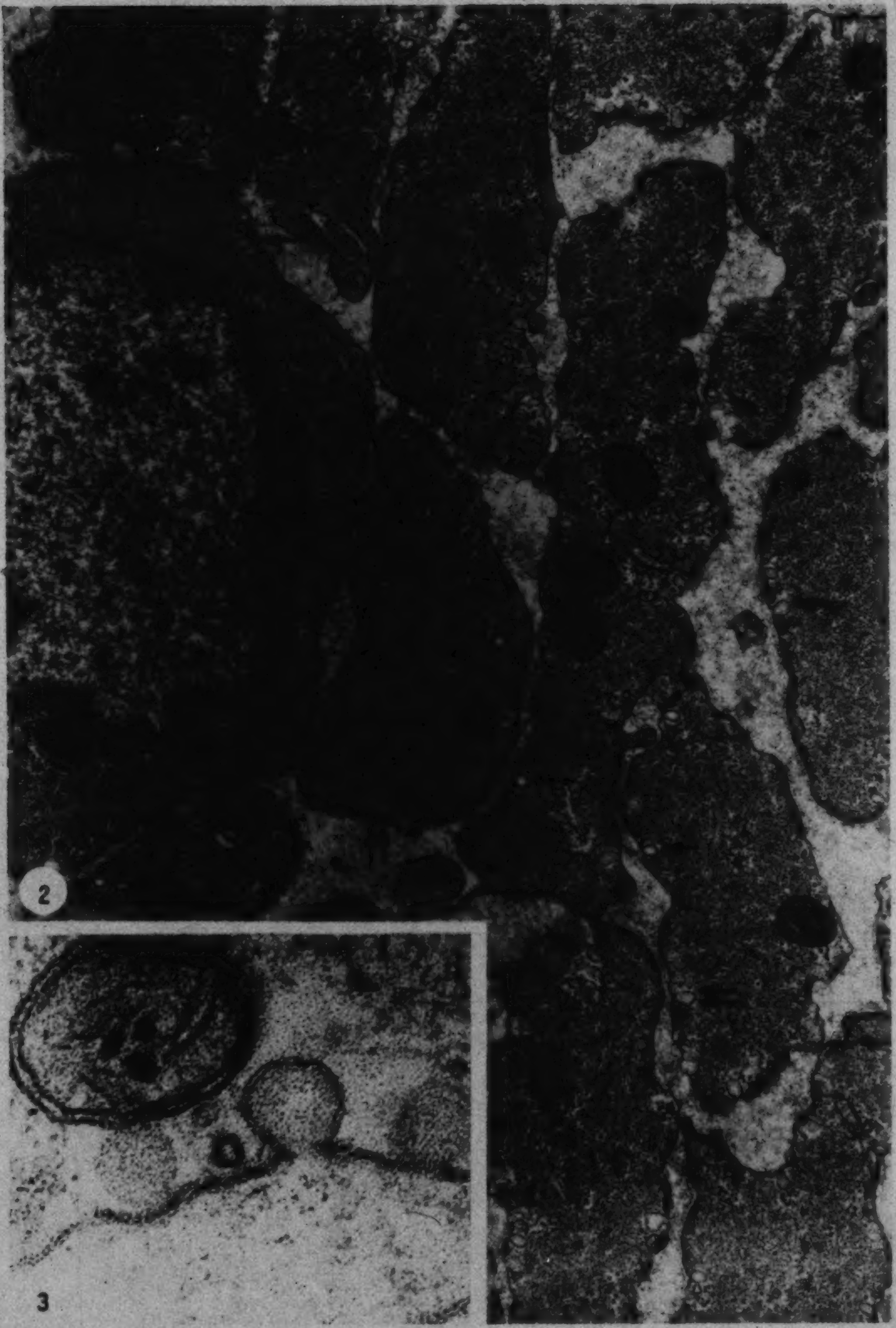

2
3

Figure 4. Smooth muscle cells fixed in 5% glutaraldehyde with 1% colloidal lanthanum nitrate. The extracellular space in some areas as shown here was completely filled with electron dense lanthanum. Lanthanum also was present in vesicles open to the exterior (v) and vesicles with no apparent opening (v'). Note the presence of particles or repeating units associated with the inner vesicular membrane (arrows). X 40,000 magnification.

Figure 5. Higher magnification of tissues shown in Figure 4. Particles were associated with the vesicular membrane (arrows) but were absent from the flat membrane. X 70,000 magnification.

4

5

ures 4 and 5). There were no particles on the flat portion of the membrane.

2. Effect of Metabolic Inhibition

Daniel and Robinson (1971c) based their vesicular hypothesis on studies of cations in whole uterine tissues which consisted of both myometrium (smooth muscle cells) and endometrium (epithelial cells). This study was designed to test their conclusions using only myometrium and to correlate the results with the ultrastructure of the cells.

IAA + DNP. Iodoacetate (IAA, a glycolytic pathway inhibitor) and 2,4-dinitrophenol (DNP, an inhibitor of oxidative phosphorylation) in concentrations of 1 mM each were used together in the incubation solution to rapidly deplete the tissues of ATP as shown by Daniel and Robinson (1971b).

Table 2 shows the effects of metabolic inhibition with iodoacetate (1 mM IAA) plus dinitrophenol (1 mM DNP) from four different experiments. There was an immediate decrease in ATP content of tissues incubated in Krebs-Ringer solution with both inhibitors. At 5 minutes the ATP content was down to 10% of the control value and it continued to decline thereafter. By 40 minutes, no ATP could be detected in inhibited tissues (<0.01% of control as judged by sensitivity of the ATP determination method). There was little change in the ATP content of control tissues incubated in Krebs-Ringer over the same time intervals (ca. 1 µmole ATP/g tissue).

The sodium content of inhibited tissues increased by 20 minutes with a simultaneous decrease in potassium content (Table 2). At approximately 30 minutes the water content of the treated tissues increased significantly. These results (ATP, Na$^+$, K$^+$ and water contents) are similar to results obtained by Daniel and Robinson (1971a,b,c) for whole uterine tissues.

The diameter of the membrane vesicles of tissues incubated only in Krebs-Ringer solution was slightly greater than 1000 Å (Table 2). There were approximately 1000 vesicles per millimeter of membrane (from cells cut in cross-section). Neither the diameter nor the number of vesicles changed consistently when incubated only in Krebs-Ringer solution.

Incubation of tissues in IAA plus DNP resulted in an increase in diameter and reduction in number of membrane vesicles by 10 minutes. The total vesicular volume/mm of membrane (average volume/vesicle x number/mm of membrane) was not significantly different from the control at 10 minutes because the size of the vesicles increased although the number decreased.

To determine if all cells react simultaneously to the effects of IAA + DNP, counts were made of cells with normal vesicles, with swollen vesicles (1000 to 1100 Å) and with no vesicles (light cells as defined in Chapter 4). Table 3 lists the number of each type of cell (% total counted) at different times after incubation with and without the inhibitors. Approximately 94% of the cells from control tissues had membrane vesicles of normal size. In tissues which were metabolically inhibited, there was a progressive shift from cells with normal vesicles through cells with swollen vesicles to cells with no

TABLE 2

Effect of iodoacetate plus dinitrophenol (IAA + DNP 1 mM each) on vesicular structure and on the contents of Na$^+$, K$^+$, ATP and H_2O of rat myometrium.

Treatment		Na$^+$ (mmoles/Kg^a dry wt.)	K$^+$ (mmoles/Kg^a dry wt.)	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membrane	Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No.)
2.5 Min	Krebs	461 (18)	327 (15)	4.15 (0.18)	Not done	1041 (31)	6.11 (0.52)	33.82 (2.24)	989 (63)	0.566 (0.051)	32.60 (2.09)
	IAA + DNP	443 (37)	318 (14)	3.88 (0.18)		1085 (33)	6.76 (0.76)	36.78 (2.49)	868 (106)	0.573 (0.666)	31.68 (3.31
5 Min	Krebs	435 (22)	316 (18)	3.95 (0.21)		1024 (17)	5.94 (0.34)	34.01 (1.42)	1268 (80)	0.759 (0.056)	43.65 (2.75)
	IAA + DNP	484 (22)	328 (16)	4.40 (0.28)	10	1160* (57)	9.60* (1.70)	45.26* (5.08)	701* (55)	0.669 (0.150)	31.43 (4.76
10 Min	Krebs	467 (31)	321 (14)	4.11 (0.25)		1144 (24)	8.10 (0.52)	41.78 (1.78)	1041 (45)	0.856 (0.075)	43.97 (3.08)
	IAA + DNP	464 (35)	261* (23)	4.34 (0.35)	6	1308* (66)	14.21* (3.49)	57.98* (8.01)	693* (55)	0.988 (0.228)	40.86 (5.51)
20 Min	Krebs	425 (28)	350 (29)	4.14 (0.18)		988 (43)	5.03 (0.75)	30.64 (2.53)	992 (82)	0.500 (0.062)	30.41 (3.57)
	IAA + DNP	517* (29)	194* (27)	4.48 (0.20)	3	1433* (83)	17.68* (3.57)	67.58* (8.32)	351* (144)	0.661 (0.137)	25.32 (3.90)
30 Min	Krebs	451 (22)	317 (14)	4.19 (0.18)		1063 (22)	6.27 (0.40)	35.47 (1.43)	997 (41)	0.600 (0.049)	33.94 (2.24)
	IAA + DNP	624* (17)	149* (4)	4.82* (0.21)	0.5	1450* (66)	15.93* (1.80)	66.05* (5.29)	352* (36)	0.561 (0.013)	23.27* (4.21)
40 Min	Krebs	488 (37)	347 (19)	4.17 (0.11)							
	IAA + DNP	623* (24)	121* (12)	4.86* (0.12)	Nil	1571 (79)	22.07 (4.28)	80.79 (9.41)	245 (30)	0.555 (0.121)	19.72 (3.29)
60 Min	Krebs	467 (21)	333 (29)	4.12 (0.18)		1012 (10)	5.49 (0.16)	32.44 (0.65)	1027 (43)	0.577 (0.032)	34.03 (1.66)
	IAA + DNP	616* (18)	85* (38)	4.65 (0.23)	Nil	1581* (78)	23.88* (4.61)	83.62* (9.24)	125* (17)	0.254* (0.039)	9.40* (1.43)
80 Min	Krebs	463 (20)	329 (29)	4.16 (0.11)		1061 (34)	4.57 (0.71)	36.19 (2.58)	1108 (31)	0.728 (0.074)	39.93 (2.84)
	IAA + DNP	650* (39)	64* (2)	4.01* (0.04)	Nil	1934* (106)	41.25* (6.00)	120.93* [illegible]	83* (17)	0.304* (0.057)	9.15* (1.80)

^{a}The Na$^+$, K$^+$ and H_2O contents are mean values from 4 experiments with S.E. in brackets . The ATP contents are from one experiment of paired controls. The control tissues all contained approximately 1 μmole ATP/gm tissue. The vesicular dimensions are the averages obtained in 16 photographs (16 photographs taken of 4 tissues at each point in time) with S.E. in brackets.

* Denotes significant differences ($p < 0.05$). Control estimates of the vesicular dimensions at 40 minutes were not done.

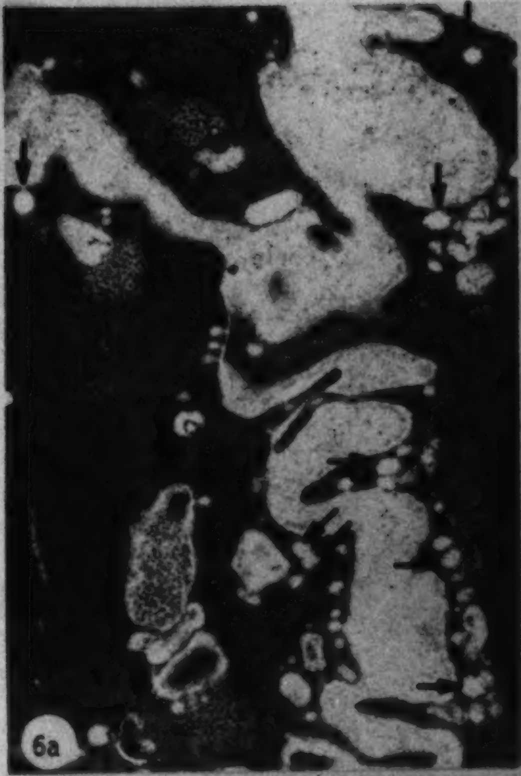
TABLE 3

Effect of IAA + DNP on the number of cells with vesicles, cells with swollen vesicles and cells with no vesicles.

Time (min)	Treatment	Total Number Cells	Normal Cells (%)	Cells Swollen Vesicles %	Cells No Vesicles %	ATP % Control
2.5	Krebs	103	94	None	6	Not Measured
	IAA + DNP	120	80	16	4	
5	Krebs	113	97	None	3	100
	IAA + DNP	119	71	14	15	10
10	Krebs	104	93	None	7	100
	IAA + DNP	131	63	32	5	6
20	Krebs	121	99	None	1	100
	IAA + DNP	105	10	48	42	3
40	Krebs	-	-	-	-	100
	IAA + DNP	100	None	54	46	0.5
60	Krebs	128	93	2	5	100
	IAA + DNP	101	None	10	90	0
80	Krebs	98	97	None	3	100
	IAA + DNP	103	None	4	96	0

The total number of cells represent smooth muscle cells counted in 16 photographs (8" x 10") from 4 separate tissues at x 24,000 magnification. Cells with less than 4 μ of membrane showing on the photograph (ca. 10 cm at x 24,000 magnification) were not counted. Control tissues at 5, 20 and 40 minutes were not done.

Figure 6. Effect of IAA + DNP (1 mM each) on the ultrastructure of uterine smooth muscle. (a) Cells from tissues after 10 minute incubation in Krebs-Ringer with the inhibitors. Note the increased size of the vesicles (arrows) as compared with those in Figure 1. Double arrows indicate two vesicles which have coalesced together. (b) Cells after 20 minutes of incubation. Note the reduced number but increased size of the vesicles (arrows) compared with those at 10 minutes. (c) Cells after 40 minutes treatment. Note that the vesicular number is decreased from those shown at 10 and 20 minutes. (d) Cells after complete ATP depletion (80 minutes). The membrane vesicles are completely absent. Magnification x 24,000.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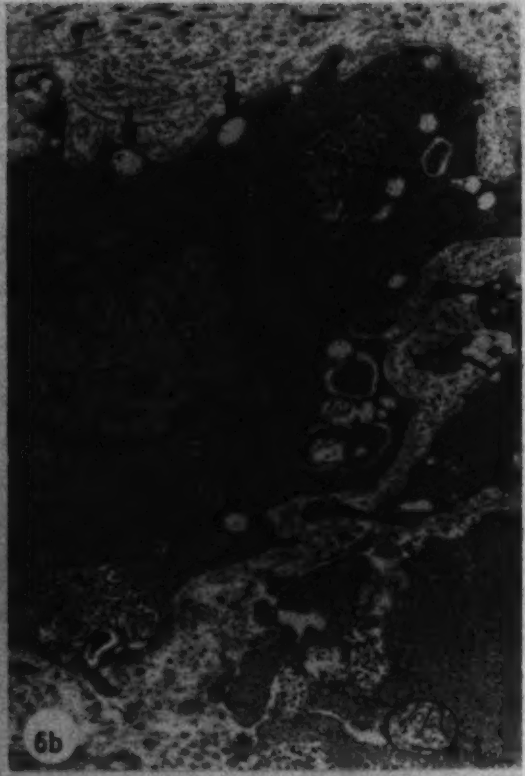
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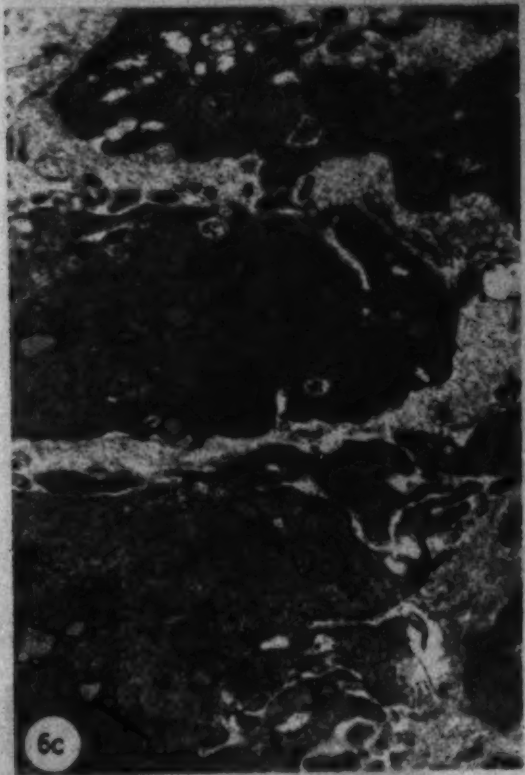
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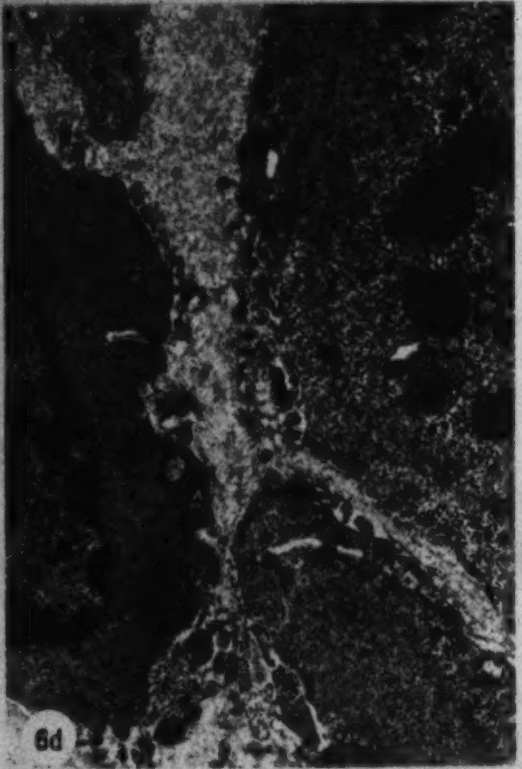

6d

vesicles. At 20 minutes, only 10% of the cells contained normal vesicles; 50% of the cells had swollen vesicles and 40% of the cells had no vesicles. After 60 minutes treatment, only 10% of the cells had vesicles and these were swollen.

Figure 6a-d shows the progressive changes which occurred in smooth muscle cells incubated in Krebs-Ringer with IAA + DNP. The membrane vesicles were swollen at 10 and 20 minutes (Figures 6a and b compared to Figure 2). At 40 minutes, there were fewer vesicles and those present were still swollen (Figure 6c). There were few vesicles present after 80 minutes of treatment (Figure 6d).

IAA. Iodoacetate used alone has been shown to decrease the ATP content of whole uterine tissues more slowly than when dinitrophenol was present (Daniel and Robinson, 1971b). If IAA alone decreases the ATP contents of myometrial tissues more slowly than when DNP is present and if ATP is responsible for the changes in cell structure (Table 2), then the structural changes should also occur more slowly with IAA.

The effects of IAA alone (1 mM) are shown in Table 4. Many effects were in general similar to results obtained when DNP was present (Table 2), although IAA alone took longer to produce the changes as predicted.

The ATP content of IAA inhibited tissues was reduced to 10% of the control at 20 minutes and no measurable ATP could be detected after 100 minutes. Significant changes in sodium and potassium content occurred by 40 minutes and water content of the treated tissues increased by approximately 100 minutes.

The number of vesicles in cells of inhibited tissues was significantly lower than the controls after 20 minutes. Unlike metabolic inhibition with IAA + DNP, inhibition with IAA alone did not increase the size of the vesicles simultaneously with reduction in their number.

The number of cells with and without vesicles is indicated in Table 5. For 120 minutes there was only a gradual change in the number of cells with vesicles (which were of normal size but reduced in numbers) to cells with no vesicles. At 120 minutes when vesicle swelling was observed, there was a marked shift from cells with normal vesicles to cells with no vesicles.

Figures 7a and b show smooth muscle cells after 40 and 80 minutes in IAA. There were fewer vesicles than the controls but relatively no change in vesicular size as indicated in Table 4. Control tissues were similar to that shown in Figure 2.

DNP. Daniel and Robinson (1971b) found that dinitrophenol reduced but did not deplete uterine tissues of ATP. If ATP is responsible for vesicle maintenance, then tissues treated with DNP might have reduced number of vesicles but DNP might not destroy the vesicles as found when IAA was used.

The effect of inhibition of oxidative phosphorylation with dinitrophenol (1 mM) is shown in Table 6.

In tissues incubated in DNP, ATP was decreased to only 65% of that of the control after 6 hours treatment. There was a slight increase in sodium content and decrease in potassium content at 2, 4 and 6 hours. However, there were no significant changes in water content.

The vesicles in cells of the 6-hour DNP treated tissues were

TABLE 4

Effect of iodoacetate (IAA 1 m$\underline{M}$)

Treatment		Na^+ (mmoles/Kg dry wt.)	K^+	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membrane	Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No.)
20 Min	Krebs	447 (10)	299 (21)	3.95 (0.17)		1093 (33)	7.14 (0.66)	38.10 (2.30)	997 (53)	0.704 (0.067)	37.77 (2.77)
	IAA	467 (10)	291 (26)	4.01 (0.10)	83.0	1020 (33)	5.77 (0.58)	33.10 (2.17)	806* (58)	0.481* (0.071)	27.29 (3.17)
40 Min	Krebs	395 (42)	331 (13)	4.01 (0.01)							
	IAA	551* (19)	278* (16)	4.35 (0.15)	10.5	1000 (31)	5.47 (0.56)	31.06 (2.07)	788 (42)	0.426 (0.046)	25.01 (2.27)
60 Min	Krebs	473 (17)	334 (25)	4.30 (0.15)		1038 (39)	5.84 (0.79)	33.82 (2.69)	964 (42)	0.563 (0.050)	32.60 (1.72)
	IAA	591* (16)	225* (11)	4.30 (0.04)	2.0	1170* (55)	9.19* (1.23)	44.40* (4.07)	636* (51)	0.549 (0.076)	27.13 (2.98)
80 Min	Krebs	472 (20)	329 (16)	4.15 (0.26)		1040 (14)	5.92 (0.23)	34.06 (0.90)	959 (50)	0.560 (0.039)	32.68 (2.02)
	IAA	633* (28)	203 * (9)	4.23 (0.16	0.3	1044 (28)	6.12 (0.51)	34.54 (1.89)	575* (53)	0.347* (0.039)	19.70* (1.96)
100 Min	Krebs	422 (16)	344 (23)	4.23 (0.25)		1025 (30)	5.63 (0.42)	33.01 (1.53)	1078 (43)	0.606 (0.051)	35.57 (2.13)
	IAA	660* (16)	164* (31)	4.53* (0.29)	Nil	1098 (38)	7.29 (0.08)	39.20 (2.82)	686* (61)	0.472* (0.047)	25.53* (2.23)
120 Min	Krebs	424 (3)	338 (17)	3.99 (0.08)		1066 (16)	6.39 (0.27)	35.00 (1.04)	945 (47)	0.595 (0.028)	33.46 (1.37)
	IAA	707* (16)	123* (10)	4.61* (0.10)	Nil	1234* (75)	11.69* (2.37)	50.64* (6.54)	332* (57)	0.275* (0.040)	13.60* (1.88)
150 Min	Krebs	480 (5)	322 (16)	3.90 (0.19)							
	IAA	874* (17)	84* (11)	4.75* (0.11)	Nil	1291 (58)	11.72 (1.60)	53.07 (4.82)	192 (27)	0.207 (0.037)	9.69 (1.51)

Results are as indicated in Table 2

TABLE 5

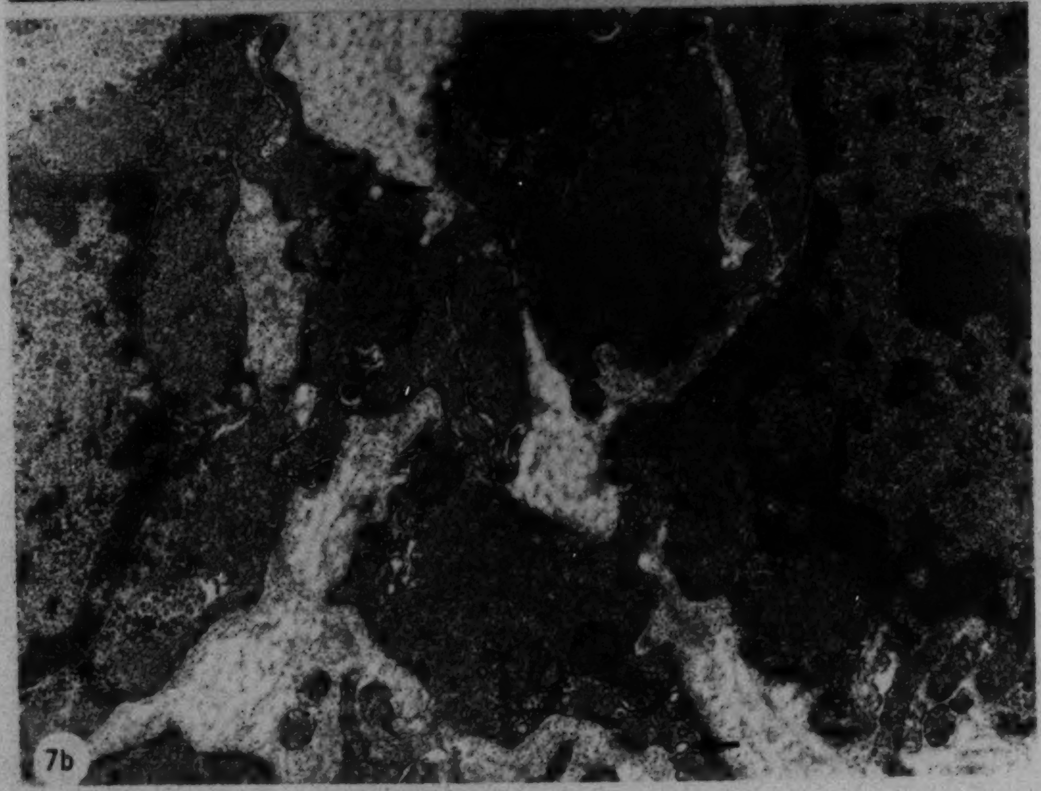
Effect of IAA on the number of smooth muscle cells with and without membrane vesicles

Time (min)	Treatment	Total Cells	Cells with Vesicles %	Cells Without Vesicles %	ATP % Control
20	Krebs	162	98	2	100
	IAA	136	94	6	83
40	Krebs	-	-	-	100
	IAA	204	84	16	10.5
60	Krebs	128	98	2	100
	IAA	149	93	7	2.0
80	Krebs	102	99	1	100
	IAA	175	82	18	0.8
100	Krebs	160	100	0	100
	IAA	128	81	19	0
120	Krebs	131	98	2	100
	IAA	146	44	56	0
150	Krebs	154	97	3	100
	IAA	164	5	95	0

Results as indicated in Table 3.

Figure 7. Effect of IAA on the ultrastructure of myometrial cells. (a) After 40 minutes inhibition. (b) After 80 minutes treatment. Note that the vesicle (arrows) population has decreased as compared with Figure 1 but the size of the vesicles remained about the same. Magnification x 24,000.

7a



7b

TABLE 6

. Effect of dinitrophenol (DNP 1 mM)

Treatment		Na^+ (mmoles/Kg dry wt.)	K^+ (mmoles/Kg dry wt.)	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membrane	Total Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No)
2 Hours	Krebs	425 (8)	348 (6)	4.25 (0.14)							
	DNP	569* (26)	264* (10)	4.47 (0.24)	103						
4 Hours	Krebs	435 (25)	366 (23)	4.19 (0.26)							
	DNP	522 (33)	224* (9)	4.00 (0.26	75						
6 Hours	Krebs	439 (30)	368 (32)	4.45 (0.27)		1138 (33)	7.96 (0.77)	41.10 (2.52)	1204 (63)	0.950 (0.093)	49.22 (3.49)
	DNP	559* (24)	244* (16)	4.40 (0.20)	65.5	1083* (22)	6.77* (0.39)	37.10* (1.46)	951* (39)	0.650* (0.051)	35.53* (2.25

Results are as indicated in Table 2

smaller than the controls. There was also a significant reduction in the number of vesicles after 6 hours DNP treatment. The diminished size and number of vesicles resulted in a significant decrease in total vesicular volume and surface area.

Figure 8 shows the ultrastructure of cells from tissues inhibited for 6 hours with DNP. The structure is similar to control tissues as shown in Figure 2.

Ethacrynic acid. Ethacrynic acid in high concentrations (1 mM) has been shown to inhibit glycolysis and oxidative phosphorylation in uterine smooth muscle and result in ATP depletion after prolonged treatment (Daniel *et al.*, 1971d). The destruction of vesicles in tissues treated with ethacrynic acid should follow ATP reduction.

In the presence of 1 mM of ethacrynic acid (Table 7), the tissue ATP content was reduced to 62% of the control at 2 hours and was almost nil at 6 hours. Tissues gained sodium and lost potassium after 2 hours treatment. The water content of tissues was significantly increased only at 6 hours.

The membrane vesicles of cells from tissues inhibited with ethacrynic acid were increased in size compared with controls and greatly decreased in number at 4 and 6 hours. Both total vesicular volume and surface area were significantly lower than the controls.

The structure of smooth muscle cells from tissues incubated for 6 hours in ethacrynic acid (Figure 9) was identical to the structure of IAA + DNP (Figure 3d) and IAA (not shown) treated tissues after ATP depletion.

TABLE 7

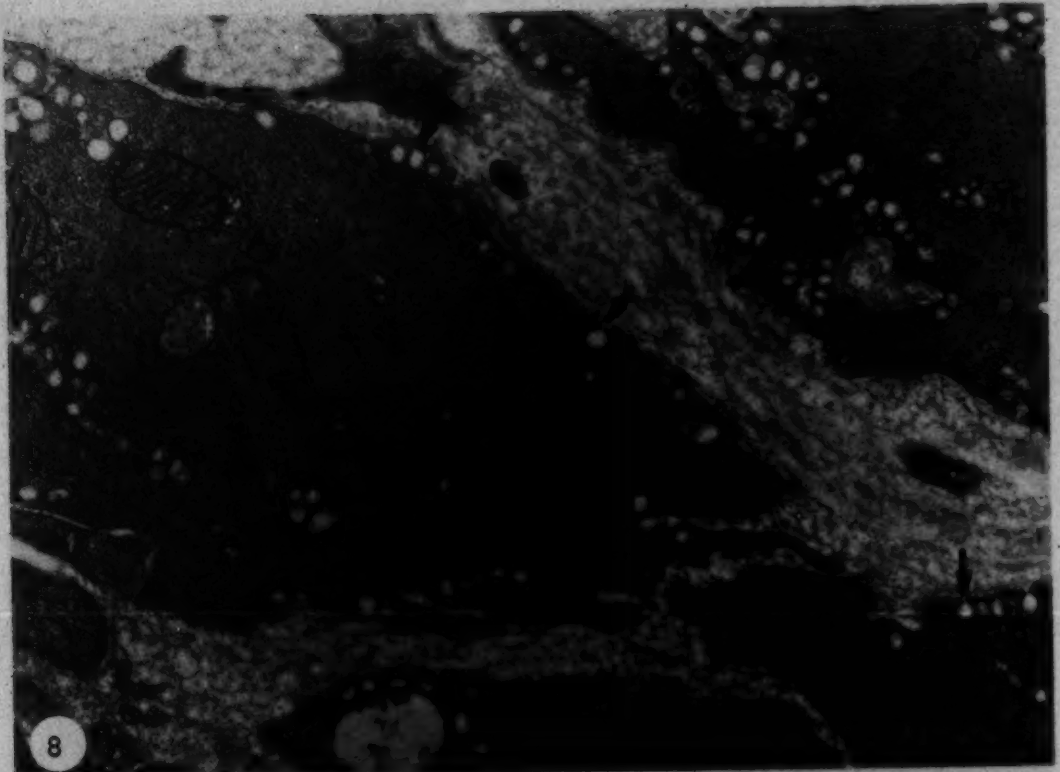
Effect of ethacrynic acid (1 m*M*)

	Treatment	Na^+ (mmoles/Kg dry wt.)	K^+ (mmoles/Kg dry wt.)	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membranes	Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No.)
2 Hr.	Krebs	457 (14)	356 (12)	4.61 (0.06)							
	Ethacrynic Acid	576* (5)	251* (18)	4.51 (0.07)	62						
4 Hr.	Krebs	459 (12)	365 (4)	4.62 (0.12)		930 (39)	4.60 (0.75)	28.24 (2.71)	1027 (92)	0.474 (0.082)	28.93 (3.66)
	Ethacrynic Acid	688* (29)	112* (30)	4.43 (0.04)	7	1218* (65)	10.61* (1.79)	48.44* (5.31)	373* (37)	0.448 (0.132)	19.50* (4.26)
6 Hr.	Krebs	448 (11)	333 (14)	4.10 (0.08)		1091 (37)	7.33 (0.83)	38.05 (2.74)	947 (42)	0.646 (0.048)	34.97 (1.74)
	Ethacrynic Acid	847* (30)	47* (5)	5.14* (21)	0.5	1806* (78)	33.32* (4.20)	105.24* (8.92)	126* (11)	0.467* (0.099)	14.25* (2.43)

Results are as indicated in Table 2

Figure 8. Ultrastructure of smooth muscle cells after treatment with DNP (1 mM) for 6 hours. The vesicles (arrows) appear similar to those indicated in Figure 1. Magnification x 24,000.

Figure 9. Effect of ethacrynic acid (1 mM) on uterine smooth muscle cells. The cells contain no vesicles and they are similar to cells depleted of ATP shown in Figure 3d. Magnification x 24,000.



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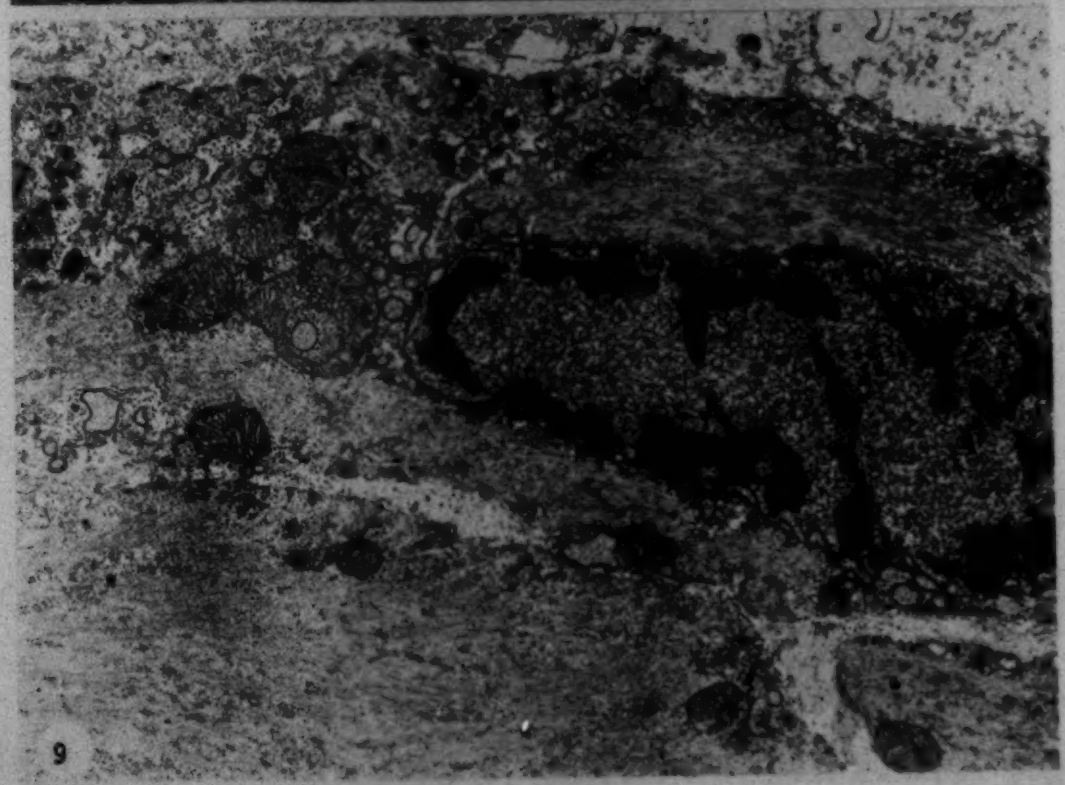

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3. *Effect of Ouabain*

Ouabain, which inhibits transport ATPase, caused Na^+ gain and K^+ loss in uterine tissues but it did not decrease ATP levels or produce water gain (Daniel and Robinson, 1971a). Thus, a second ouabain-insensitive volume pump was postulated. If this pump is associated with the vesicles, then ouabain should have no effect on the vesicles.

Ouabain had little effect on the ATP content of tissues measured at 2, 4 and 6 hours (Table 8). Ouabain only slightly increased the sodium content of tissues in relation to dry weight (ca. 25% increase), whereas less than one-fourth of the initial potassium remained. Water contents of ouabain-inhibited tissues were reduced, possibly accounting for the failure of sodium gain to equal potassium loss.

Treatment with ouabain for 2 hours did increase the number of vesicles of smooth muscle cells; however, there was no significant increase at 6 hours (Table 8). The average size of the vesicles was increased significantly at 6 hours. The reason for the increased vesicular size was found to be due to two populations of vesicles after ouabain inhibition; the normal sized vesicles (ca. 1000 Å diameter) and vesicles of increased size (ca. 1800 Å diameter). The larger vesicles were found both on the surface and intracellularly as shown in Figure 10. The larger vesicles had no visible connections to the extracellular space.

Since ouabain causes contracture of uterine smooth muscle, tissues were incubated with ouabain in the presence of *l*-adrenaline (2 μg/ml, 10 μM). Adrenaline has been shown to inhibit ouabain pro-

TABLE 8

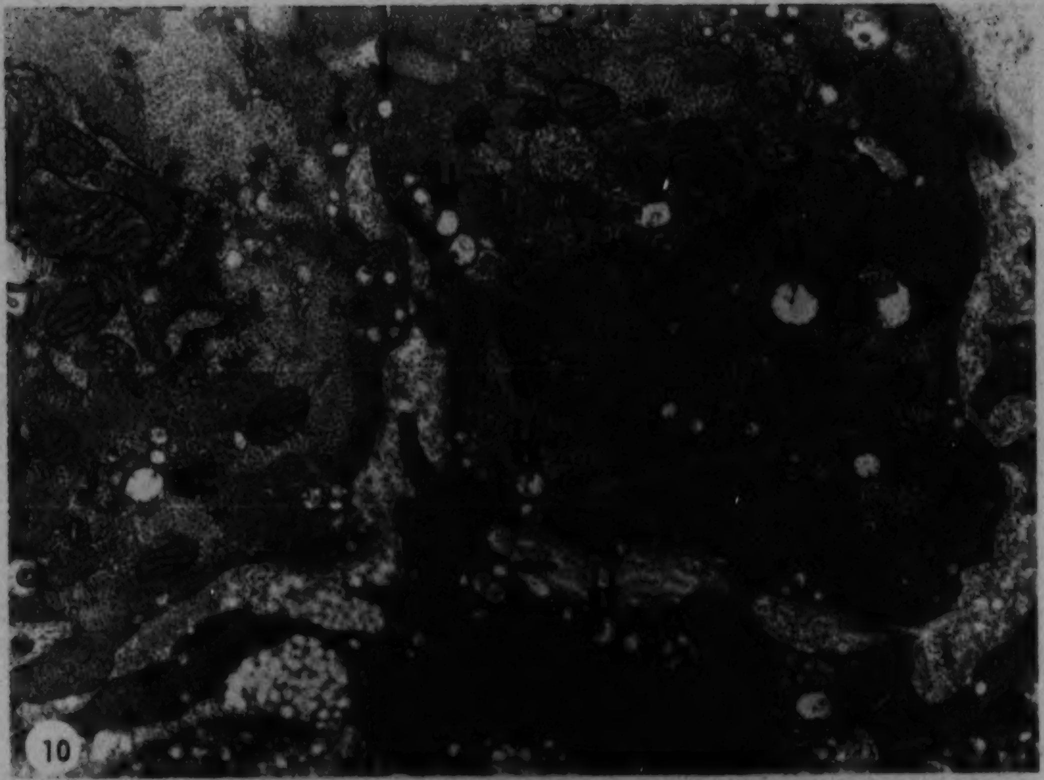
Effect of ouabain (1 mM)

	Treatment	Na^+ (mmoles/Kg dry wt.)	K^+ (mmoles/Kg dry wt.)	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membranes	Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No.)
2 Hr.	Krebs	432 (30)	380 (42)	4.35 (0.18)		1142 (47)	8.28 (0.9)	41.73 (3.20)	852 (41)	0.723 (0.099)	36.28 (3.85)
	Ouabain + Adren. (2 μg/ml)	523 (27)	105* (20)	3.32* (0.21)		1067 (55)	6.95 (1.18)	36.86 (4.01)	866 (64)	0.654 (0.162)	33.64 (6.10)
2 Hr.	Krebs	444 (22)	329 (40)	3.98 (0.12)		988 (33)	5.27 (0.54)	31.12 (2.10)	1090 (60)	0.579 (0.053)	34.78 (2.68)
	Ouabain	517 (25)	82* (12)	3.02* (0.08)	90	1075 (46)	7.02 (0.97)	37.24 (3.31)	1246 * (70)	0.856* (0.106)	45.97* (4.33)
4 Hr.	Krebs	428 (20)	362 (48)	4.27 (0.19)							
	Ouabain	561* (24)	98* (14)	3.50* (0.21)	95						
6 Hr.	Krebs	459 (58)	356 (18)	4.34 (0.18)		1117 (23)	7.42 (0.44)	39.46 (1.59)	1118 (80)	0.829 (0.076)	44.09 (3.63)
	Ouabain	523 (40)	72* (10)	3.22* (0.20)	90	1275* (28)	11.08* (0.79)	51.45* (2.36)	1028 (48)	1.150* (0.106)	53.22* (3.79)

Results are as indicated in Table 2

Figure 10. Uterine smooth muscle cells after 6 hours treatment with ouabain (1 mM). Note the presence of two types of vesicles. Smaller vesicles like those found in control tissues (arrows) and larger surface and intracellular vesicles (double arrows). Magnification x 24,000.

Figure 11. Smooth muscle cells from tissue made Na^+-rich by overnight incubation in K^+-free Krebs-Ringer at 5°C. The membrane vesicles (arrows) were reduced in number but otherwise the cells appeared normal compared with tissues incubated in Krebs-Ringer at 25°C. Magnification x 24,000.



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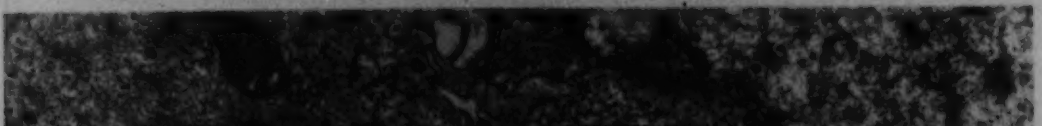

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duced contraction but adrenaline does not inhibit the effect of ouabain on sodium transport in the myometrium (Daniel, 1964). In the presence of adrenaline, the effects of ouabain on cations, water content and ultrastructure were similar to those obtained in the absence of adrenaline at two hours (Table 8).

4. *Sodium-rich Tissue*

Previous studies indicated that when tissues were made sodium-rich at 5°C they gained Na^+ and lost K^+ but they did not always swell (Daniel and Robinson, 1970c; Rangachari, 1972; Rangachari *et al.*, 1972). When sodium-rich tissues were rewarmed in K^+-free Krebs-Ringer at 37°C they lost Na^+, Cl^- and water and the tissues contracted. Rangachari (1972) further noted that iodoacetamide and Ca^{++}-free solutions, which had no effect on ATP levels, inhibited the loss of sodium, water and contraction which occurred during rewarming. Rangachari *et al.* (1972) suggested that a mechanochemical system associated with the membrane might be involved in the sodium and water loss. If the membrane vesicles of smooth muscle cells are sites of a mechanochemical system, then rewarmed Na^+-rich tissues should have altered vesicle structure or number than those found in cold Na^+-rich tissues. This alteration in vesicle size or number could be prevented by iodoacetamide or Ca^{++}-free solutions. The object of studying Na^+-rich tissues was to test the above hypothesis.

Pieces of sodium-rich tissues for electron microscopy were taken from the same uterine horn (cervical end) after 24 hours incubation at 5°C and after rewarming the tissue for 20 minutes at 37°C (Groups A & B, Tables 9, 12, 13). After rewarming, the remaining piece of tissue was used to measure cation and water contents. The paired horn was handled in a similar manner but treated before and during the rewarming period (Groups C & D, Tables 9, 12, 13).

Tissues made Na^+-rich by incubation in K^+-free solution at 5°C, gained water (Group A, Tables 9, 12, 13). The same tissues lost water upon rewarming in the same solutions (Group B, Tables 9, 12, 13). These results are similar to those obtained by Daniel and Robinson (1970) and Rangachari (1972).

The ATP content of sodium-rich tissues was reduced to 72% that of the controls (fresh tissue). The ATP content of rewarmed tissues was not measured; however, Rangachari (1972) found that the levels were not significantly different from those measured before rewarming.

The vesicular diameter of Na^+-rich tissues fixed for electron microscopy before rewarming was sometimes significantly larger than that of the rewarmed tissues (Group B *vs*.A, Tables 9, 12, 13).

The number of vesicles along the cell membrane of sodium-rich tissues was always approximately one-half that found in control fresh tissues (Group A, Tables 9, 12, 13 *vs*. Table 2). Rewarming caused a significant increase (30 to 50%) in the number of vesicles when compared with cold sodium-rich tissues but the resultant number of vesicles was usually less than that found in control fresh tissues (Group B, Tables 9, 12, 13 *vs*. Table 2).

Thus, in the cold there was little change in vesicle size but the number of vesicles per unit length of membrane was less. Rewarming, under conditions in which the Na^+-K^+-ATPase was inhibited (K^+-free solutions), caused a significant increase in vesicle number.

Iodoacetamide. There was significantly more Na^+ in tissues rewarmed with iodoacetamide (Group D *vs.* B, Table 9). Iodoacetamide also inhibited the water lost during rewarming.

Rewarming of tissues with IAAmide caused a significant increase in size, number and volume of the vesicles from measurements of vesicles in tissues taken at 5°C with IAAmide, but the vesicle size was not different from tissues rewarmed without IAAmide.

Figure 11 shows the structure of cells from sodium-rich tissue. There was no apparent damage to cells after 24 hours incubation at 5°C. The mitochondria were all in the condensed state as described by Hackenbrock (1966) and neither the endoplasmic nor nuclear membranes were swollen. The structure of rewarmed tissues was similar to that of control fresh tissues incubated at 25°C (Figure 2). The presence of IAAmide did not change the structure of either the cold or rewarmed tissues.

Ca^{++}-free. When tissues were incubated overnight in Ca^{++}-free solutions, the tissues gained water compared to fresh weights (Table 10). The presence of Ca^{++} was necessary for the loss of Na^+ and water when the tissues were rewarmed.

The vesicles increased significantly in number in tissues rewarmed in solutions containing Ca^{++} (Group C *vs.* D). Without Ca^{++} in the rewarming solution, the vesicles were greater in size (Group A *vs.* B) as when IAAmide was present in the rewarming solution (Table 9, Group C *vs.* D). Thus, Ca^{++} was necessary for the Na^+-K^+-ATPase-independent Na^+ and water loss as well as the increase in vesicle number upon rewarming of Na^+-rich tissues.

TABLE 9

Effect of cold (5°C) and rewarming (37°C) in K^+-free Krebs-Ringer solution in the presence of iodoacetamide (0.1 mM IAAmide).

Treatment	Na^+ (mmoles/Kg dry wt.)	K^+ (mmoles/Kg dry wt.)	H_2O (g/g solid)[a]	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membranes	Volume Vesicles/mm (Vol. x No.)	Surface Area/mm (S.A. x No.)
A. Na^+-Rich	-	-	(104.6±0.9)	72 (2.1)	1160 (76)	9.86 (2.24)	44.95 (6.33)	575 (51)	0.349 (0.630)	23.51 (2.73)
B. Na^+-Rich Rewarm 20'	739 (27)	trace	4.13 (96.0±0.5)*	-	1253 (58)	11.16 (1.40)	50.77 (4.34)	812 (58)*	0.913 (0.147)*	41.37 (5.15)*
C. Na^+-Rich IAAmide 30'	-	-	(105.6±1.5)	-	1070 (58)	7.25 (1.58)	37.25 (5.49)	583 (57)	0.400 (0.084)	21.03 (2.71)
D. Na^+-Rich + IAAmide Rewarm 20'	953† (58)	trace	5.45† (104.2±1.9)†	-	1241 (42)	10.50 (1.70)*	49.19) (3.37)*	777 (48)*	0.840 (0.108)*	37.75 (3.11)*

Tissues were made Na^+-rich in cold (5°C) K^+-free solution and rewarmed (37°C) for 20 minutes in K^+-free solution. Iodoacetamide (0.1 mM) was added to the cold solution 30 minutes before rewarming. Tissues A and B were from the same uterine horns (n = 4 observations) taken before (A) and after rewarming (B) for electron microscopy. Tissues C and D were from the opposite paired horns taken before (C) and after rewarming (D). The tissues remaining after rewarming were analyzed for cations. The ATP contents are from earlier experiments (n = 8).

[a] H_2O contents in brackets indicate the water contents expressed as % of fresh weight ± S.E.

*Indicates significant differences between tissues from the same uterine horns.

†Designates significant differences between paired tissues.
Results as indicated in Table 2.

TABLE 10

Effect of cold (5°C) and rewarming (37°C) in K^+-free Krebs-Ringer without Ca^{++}.

Treatment	Na^+ (mmoles/Kg dry wt.)	K^+ (mmoles/Kg dry wt.)	H_2O (g/g solid)	ATP (% control)	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membranes	Volume Vesicles/mm (Vol. x No.)	Surface Area per mm (S.A. x No.)
Na^+-Rich Ca^{++}-free A	-	-	(121.6±3.31)		1078 (53)	7.34 (1.38)	37.85 (4.22)	338 (32)	0.225 (0.031)	12.08 (1.18)
Na^+-Rich Ca^{++}-free Rewarm B	838 (36)	trace	4.81±0.10 (123.0±2.7)		1272* (70)	12.43* (2.28)	53.27* (6.25)	403 (49)	0.435* (0.089)	19.55* (2.82)
Na^+-Rich Ca^{++}-free C	-	-	(119.9±4.0)		1023 (59)	6.64 (1.69)	34.60 (4.86)	406 (37)	0.250 (0.053)	13.42 (1.66)
Na^+-Rich Rewarm + Ca^{++}	688 (28)	trace	3.81±0.25 † (108.8±3.1)*		1100 (48)	7.63 (1.17)	39.14 (3.71)	570* (31)	0.456 * (0.087)	22.97* (3.00)

Results and experimental design as indicated in Table 9. Tissues were made Na^+-rich in K^+-free, Ca^{++}-free Krebs-Ringer at 5°C and rewarmed in the same solution with (Group D) and without 2.5 mM Ca^{++} (Group B).

Ouabain. Rangachari et al. (1972) found that Na^+-rich tissues rewarmed under conditions of Na^+-K^+-ATPase activity (i.e., in normal Krebs-Ringer solution which contained K^+) also lost water but the tissues did not contract. Ouabain did not affect the water loss. Rangachari proposed that the loss of water was due to a mechanochemical event in the membrane unrelated to either activity of Na^+-K^+-ATPase or contraction of the entire tissue.

If the above hypothesis is true and if the vesicles are the loci of the mechanochemical system, Na^+-rich tissues rewarmed ± ouabain in normal Krebs-Ringer solution should be identical to tissues rewarmed in K^+-free solutions.

There was no difference between sodium-rich tissues rewarmed in normal Krebs-Ringer with ouabain and tissues rewarmed without ouabain (table 11). There was a significant inhibition in the uptake of potassium in tissues rewarmed with ouabain. Water contents of both groups of rewarmed tissues were decreased compared with tissues in the cold. However, when ouabain was present during rewarming (Group D), the water contents of tissues were even less than those tissues rewarmed without ouabain (Group B).

The vesicular diameter was greater in sodium-rich tissues rewarmed in normal Krebs-Ringer (K^+-containing) than vesicles of Na-rich tissues which were not rewarmed, but the number was less, resulting in little change in total vesicular volume or surface area (Group A *vs.* B, Table 11). The presence of ouabain in the rewarming solution caused an increase in size and an increase in number of vesicles. In fact, ouabain increased the number of vesicles to more than twice as many as present in tissues rewarmed without ouabain (Group B *vs.* D).

TABLE 11

Effect of cold (5°C) and rewarming (37°C) in normal Krebs-Ringer solution with ouabain.

Treatment	Na^+ (mmoles/Kg dry wt.)	K^+	H_2O % of Fresh weight	Average Vesicular Diameter (A°)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-3}$)	Number Vesicles (no./mm)	Volume Per mm (Vol. x No.)	Surface Area Per mm (S.A. x No.)
A. Na^+-Rich Cold	-	-	(107.4±0.9)	989 (42)	5.53 (0.82)	31.62 (2.93)	463 (45)	0.271 (0.059)	15.13 (2.41)
B. Na^+-Rich Rewarm 20'	596 (39)	152 (9.3)	3.9±0.10 (94.1±0.2)*	1304* (68)	12.83* (1.65)	55.40* (5.21)	324* (76)	0.384 (0.102)	16.90 (4.02)
C. Na^+-Rich Cold + Ouabain 20'	-	-	(104.8±[illegible].7)	1146 (58)	8.81 (1.45)†	42.80 (4.55)†	565 (66)	0.415 (0.058)	21.54 (2.42)
D. Na^+-Rich Rewarm + Ouabain 20'	595 (22.6)	24† (4.3)	3.57±0.12† (88.7 +2.1)*	1236 (50)	10.63 (1.16)	49.27 (3.78)	831* (60)†	0.972* (0.105)†	40.60* (3.89)†

Data as indicated in Table 9; experimental design as in Table 9. Tissues were made Na^+-rich in cold K^+-free solution and rewarmed in normal Krebs-Ringer solution at 37°C. Ouabain (1 mM) was added to the cold solution 20 minutes before rewarming.

The total vesicular volume and surface area were also twice as great when ouabain was present. The vesicle size may have already increased with ouabain in the cold (Group C *vs.* A). The values obtained when ouabain was present in the rewarming solution (Table 11, Group C *vs.* D) were similar to values obtained when tissues were rewarmed in K^+-free solutions (Table 9, Group A *vs.* B). Thus, inhibition of Na^+-K^+-ATPase during rewarming with either ouabain or K^+-free solutions caused increase in vesicle number relative to control Na^+-rich tissues at 5°C. Rewarming of tissues under conditions of Na^+-K^+-ATPase activity did not produce the expected increase in vesicle number.

The structure of rewarmed tissues treated with ouabain was similar to fresh tissues treated with ouabain (Figure 10). There were large intracellular vesicles as noted when fresh tissue was treated with ouabain.

5. *Substitution of other cations*

To see if other cations would substitute for Na+, tissues were incubated at 5°C for 24 hours in solutions enriched in either K^+ or Li^+. These tissues were then rewarmed at 37°C for 20 minutes in the same solutions.

Like tissues made Na^+-rich in the cold and rewarmed in K^+-free solutions, tissues made K^+-rich in the cold and rewarmed in K^+-enriched solution also lost water and the vesicular number and volume increased (Group A *vs.* B, C *vs* D; Table 12).

TABLE 12

Effect of cold (5°C) and rewarming (37°C) in K^+-Krebs-Ringer.

Treatment	Na (mmoles/Kg dry wt)	K^+ (mmoles/Kg dry wt)	H_2O (g/g solids)	Average Vesicular Diameter (A)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-2}$)	Number Vesicles/mm Membranes	Volume Vesicles/mm Membranes	Surface Area Vesicles/mm Membranes
Na^+-Rich A	-	-	103.1±2.0	770 (28)	2.55 (0.35)	19.02 (1.55)	442 (24)	0.114 (0.017)	8.46 (0.84)
Na^+-Rich Rewarm 20' B	750 (35)	trace	4.05 (0.1) 88.5±3.6*	909* (35)	4.19* (0.45)	26.53* (1.99)	1058* (73)	0.442 (0.056)	28.76* (2.88)
K^+-Rich C	-	-	100.9±2.7	778 (25)	2.58 (0.28)	19.33 (1.32)	480 (50)	0.135 (0.027)	9.81 (1.55)
K^+-Rich Rewarm 20' D	trace	739 (60)	4.12 (0.2) 87.2±1.3*	853* (27)	3.37* (0.32)	23.18 (1.45)	794 (67)	0.276 (0.042)	18.74* (2.34)

Results and experimental design as indicated in Table 9. Tissues A and B were from myometria taken before (A) and after rewarming in K^+-free solution. Tissues C and D were from the opposite paired horns made K^+-rich in Na^+-free (K^+-substituted for Na^+) solutions. Tissues were removed before (C) and after rewarming (D) in Na^+- free solution.

TABLE 13

Effect of cold (5°C) and rewarming (37°C) in Li^+-Krebs solution.

Treatment	Na^+ (mmoles/kg dry wt)	Li^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)	H_2O (g/g solids)	Average Vesicular Diameter (Å)	Average Vesicular Volume ($\mu^3 \times 10^{-4}$)	Average Vesicular Surface Area ($\mu^2 \times 10^{-3}$)	Number Vesicles/mm Membrane	Volume Vesicles/mm Membrane	Surface Area Vesicles per min Membrane
Na^+-Rich A	-	-	-	104.6±1.6	839 (31)	3.27 (0.37)	22.52 (1.66)	382 (43)	0.119 (0.019)	8.33 (1.08)
Na^+-Rich Rewarm 20' B	722 (24)	-	trace	3.59(0.28) 96.7±0.8 *	910 (25)	4.07 (0.34)	26.28 (1.47)	958* (68)	0.400* (0.052)	25.63* (2.73)
Li^+-Rich C	-	-	-	108.7±1.5	737 (35)	2.33 (0.45)	17.63 (1.96)	467 (52)	0.103 (0.012)	7.53 (0.83)
Li^+-Rich Rewarm 20' D	trace	918 (33)	trace	6.14 (0.21) 111.8±1.6	1137* (82)	966* (2.51)	44.78* (6.87)	283* (37)	0.244* (0.060)	11.53* (2.15)

Results and experimental design as in Table 9.

Tissues A and B were incubated in the cold and tissues B were rewarmed in K^+-free Krebs solution.

Tissues C and D were incubated in the cold and tissues D were rewarmed in Li^+-Krebs solution (Li^+ substituted for Na^+ and K^+.

As compared to Na^+-rich tissues, Li^+-rich tissues did not lose water when rewarmed, the vesicular size increased significantly and the vesicular number decreased significantly (Table 13).

6. *Effects of Various Other Treatments*

Tissues were also examined qualitatively after various other treatments (Table 14).

To determine if the movements of Na^+ and K^+ alone without ATP depletion were responsible for vesicle destruction, tissues were made K^+-rich in a K^+-Krebs-Ringer solution (K^+-substituted for Na^+, Group A). The vesicles in tissues made K^+-rich were similar to tissues incubated in normal Krebs-Ringer solution, The addition of IAA (1 mM) to the solution for 2 hours depleted the tissues of ATP, caused a gain in water and destroyed the vesicles.

Tissues inhibited with IAA (1 mM) for 30 minutes and then incubated in pyruvate for 2 hours were studied to see if pyruvate could overcome the glycolytic blockade produced with IAA (Group B). Pyruvate, but not glucose, was able to maintain the ATP content of tissues inhibited with IAA at approximately the same level of ATP the tissues contained after 30 minutes inhibition with IAA alone (see Table 4). There were vesicles present in the tissues incubated with pyruvate but none present in the tissues incubated in glucose.

Tissues were incubated in Krebs-Ringer without glucose but with DNP (1 mM) or bubbled with nitrogen (95% N_2 - 5% CO_2) to see the relationship between ATP levels and the presence of vesicles in the tissue (Group C & D). The ATP content of DNP treated tissues was reduced to 20% that of the control after 4 hours. Nitrogen reduced the ATP content to 32% of the control level after 8 hours. Neither substrate depletion with DNP nor with nitrogen asphyxia produced any changes in vesicular structure.

Tissues incubated in Krebs-Ringer with IAA + DNP (1 mM each) + ATP (1 mM) were studied to see if exogenous ATP could support the membrane vesicles (Group E). The presence of ATP with the inhibitors did not alter the electrolyte or vesicular changes.

TABLE 14

Effect of various other treatments

Treatment		Time (Hours)	ATP (% control)	Electrolytes	H_2O (% fresh weight)	Membrane Vesicles
A. K^+-Krebs + IAA		2	0	↓ Na^+, K^+ ↑	131	Absent
Control (K^+-Krebs)		2	100	↓ Na^+, K^+ ↑	95	Present
B. IAA + pyruvate	30 min IAA → 2 pyruvate		10			Present
	30 min IAA → 2 glucose		0			Absent
C. DNP + glucose-free*		4	20			Present
Control (in normal Krebs)		4	100			Present
D. Nitrogen + glucose-free*		8	32			Present
Control (in normal Krebs)		8	100			Present
E. IAA + DNP + ATP		1		↑ Na^+, ↓ K^+	120	Absent
IAA + DNP		1	—	↑ Na^+, ↓ K^+	124	Absent
ATP		1			96	Present
Control		1			100	Present

Results are from one or two paired control experiments.

Group A. Tissues were incubated in a K^+-Krebs-Ringer (K^+ substituted for Na^+) for 1 hour at which time one horn was transferred to the same solution containing IAA (1 mM) for an additional 2 hours.

Group B. Fresh tissues were incubated for 30 minutes in Krebs-Ringer + IAA (1 mM) at which time one horn was transferred to Krebs-Ringer with pyruvate (50 mM) substituted for glucose and then the other horn was transferred to normal Krebs-Ringer solution.

Group C. Fresh tissues were inhibited in Krebs-Ringer solution with DNP (1 mM) which contained no glucose.

Group D. Inhibition of fresh tissues was with a glucose-free Krebs-Ringer aerated with 95% N_2-5% CO_2.

Group E. Fresh tissues were incubated with IAA+DNP (1 mM each) with and without ATP (1 mM) or with ATP alone.

* Sucrose was substituted for glucose in Krebs-Ringer solution.

D. Discussion

1. *Correlation of results with the vesicular Hypothesis*

The foregoing ultrastructural study supports the vesicular hypothesis proposed by Goodford *et al.* (1968) and Daniel and Robinson (1971c). This study presents evidence which indicates that the membrane vesicles have several properties in common with the volume pump described by Daniel and Robinson (1971a, b, c) and Rangachari *et al.* (1972). These properties include the following:

(a) The volume pump requires ATP; the vesicles are also ATP-dependent in that they disappear following depletion of ATP.

(b) Inhibition of metabolism is accompanied by cation changes and loss of volume control; after metabolic inhibition, changes in vesicular structure precede or are accompanied by changes in cations and volume.

(c) Treatment of fresh tissues with ouabain does not inhibit the volume pump; ouabain does not affect the number of vesicles in fresh tissues.

(d) A gain in water occurs when tissues are made Na^+-rich in the cold, and these tissues lose water when rewarmed; the vesicle number is reduced in tissues incubated in the cold and the vesicle number increases when these tissues are rewarmed under conditions thought to inhibit Na^+-K^+-ATPase.

(e) The loss of water from Na^+-rich tissues during rewarming is Ca^{++}-dependent; the increase in vesicle number during rewarming also requires Ca^{++}.

(f) The volume pump is not specific for Na^+; the increase in vesicle number upon rewarming of tissues incubated in the cold is not dependent upon Na^+ (K^+, but not Li^+, will substitute for Na^+).

ATP-dependence. Complete depletion of ATP with metabolism inhibitors resulted in complete loss of normal sized vesicles in smooth muscle. Inhibitors which reduced the ATP content of tissues to below 50% of the normal level reduced the number of membrane vesicles. Cooling also decreased the number of vesicles with the level of ATP about 75%. These findings support the concept that a certain amount of ATP is necessary for vesicle maintenance. However, the correlation between vesicle number and ATP content of tissues is more complex than originally believed.

To analyze the relationship between ATP content and vesicle number in tissues, initial considerations of possible models is helpful. For any individual cell, vesicle number can be assumed to be some function of the ATP level such that as the ATP level falls, vesicle number falls. This relationship may involve (a) a step; i.e., a reduction below threshold of ATP may be required before vesicle change occurs or (b) a lag; i.e., a time delay between ATP depletion and its effect on vesicle number or both. When dealing with a tissue composed of a multitude of cells, the decrease in ATP content of the tissue with time could occur in different ways.

(a) A simultaneous decrease of ATP in all cells (all cells are the same initially and react identically).

Figure 12. The change in vesicular number with change in ATP. This figure is a composite of data obtained from all experiments with fresh tissues. This curve was found to fit these points as analyzed by linear regression analysis.

Figure 13. The change in vesicular volume with change in ATP. Data from fresh tissues as in Figure 12.

Figure 12

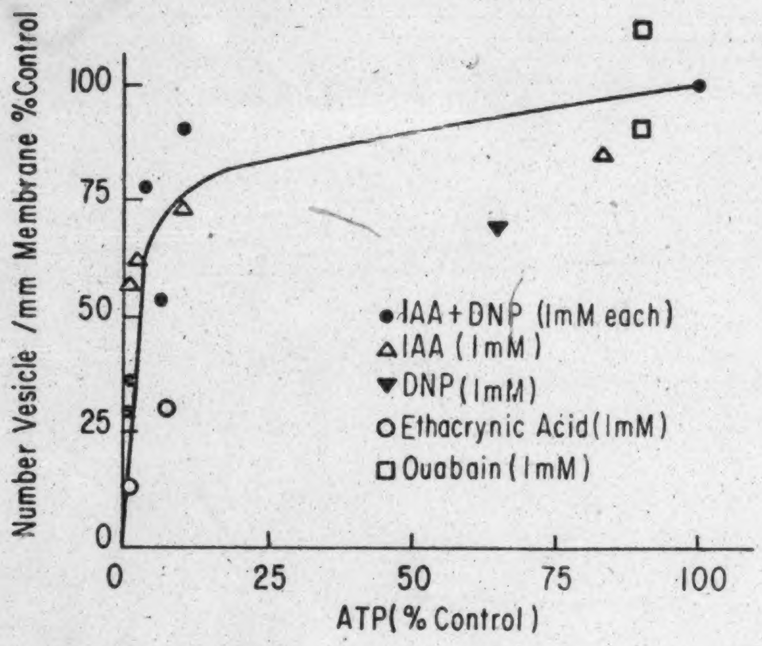


Figure 13

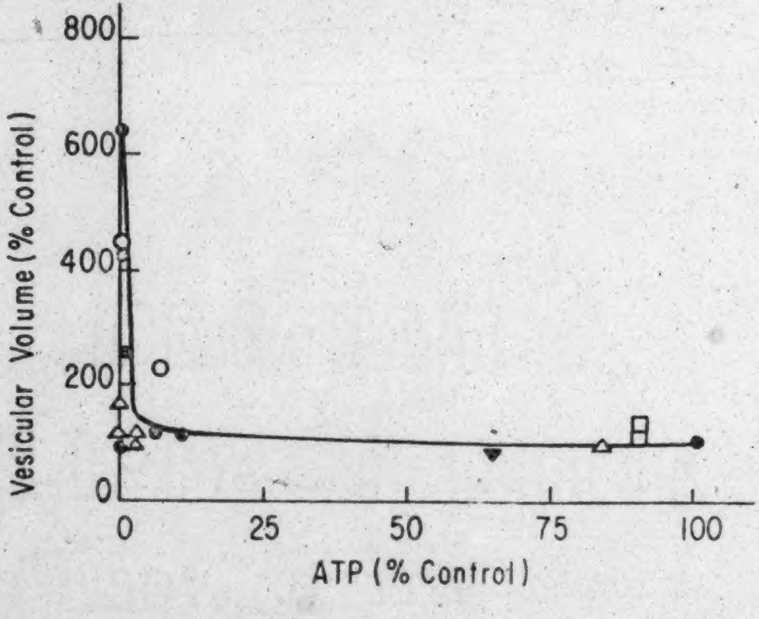


Figure 14. Effect of metabolic inhibition with IAA + DNP on ATP and vesicle changes. Data from Tables 2 and 3.

Figure 15. Effect of metabolic inhibition with IAA on ATP and vesicular changes. Data from Tables 3 and 4.

Figure 14. IAA + DNP (1 mM each)

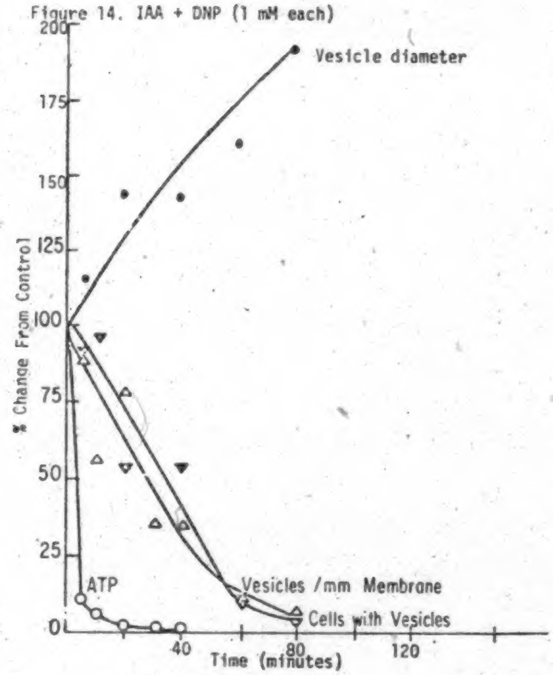
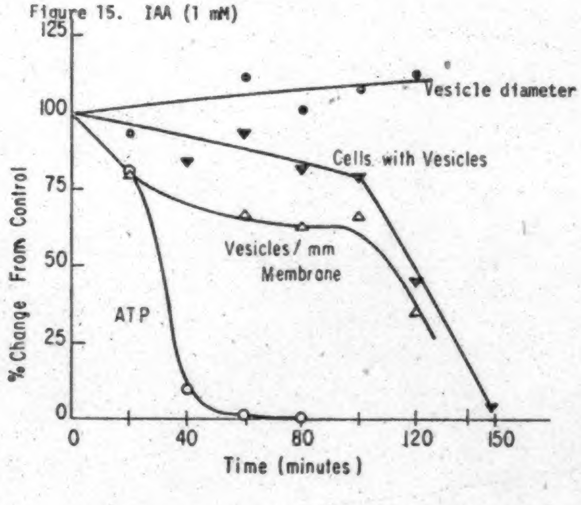


Figure 15. IAA (1 mM)



(b) A differential effect in which some cells contain their normal or a lower complement of ATP whereas others contain none due to either different sensitivities of the cells to metabolic inhibition or due to initial variation among cells in ATP content.

The threshold level of ATP necessary to maintain both vesicle number and vesicle size was approximately 10% of the control levels (Figures 12 and 13). These figures were drawn using data from all the experiments with fresh tissues in which ATP level was altered.

Shortly after complete ATP depletion, all smooth muscle cells contained essentially no vesicles (Tables 2, 3, 4 and 5). If a simultaneous decrease of ATP occurred in all cells, a decrease in ATP content below the threshold level should produce decreases in vesicular number shared by all cells which should precede the decline in the number of cells with vesicles. If the cells contained different amounts of ATP initially, the proportion of cells with no vesicles ought to parallel the decrease in numbers of vesicles. The necessity for studying the alternatives is obvious if a relationship between ATP and vesicle number is to be made.

There was a rapid decrease in ATP content of tissues inhibited with either IAA + DNP or IAA alone (Figures 14 and 15). This was followed, after a lag period which was longer in IAA than in IAA + DNP, by a simultaneous reduction in the number of vesicles per unit length of membrane and changes in the percentage of cells with vesicles. These results are consistent with a postulate that cells differ in respect to initial ATP contents and react correspondingly when inhibited metabolically. Another model one could postulate is that the ATP levels are constant in all cells at time zero and sensitivities and delays vary in different cells when inhibited.

The lag period between ATP and vesicle depletion could be accounted for if ATP depletion causes a chain of events, each event requiring a finite time, the sum of which is a lag before the observed disappearance of vesicles. The difference in lag periods seen after inhibition with IAA + DNP and IAA alone could be related to swelling and destruction of vesicles which occurs only with IAA + DNP.

Keeping tissues for 24 hours in K^+-free Krebs-Ringer at 5°C decreased the number of vesicles to one-half that found in control tissues incubated at 25°C (Tables 9, 12, 13). This procedure did not decrease the ATP contents below the threshold of ATP necessary for vesicle maintenance found with metabolic inhibitors (70% *vs.*10%). Thus, the ATP content cannot account for the decrease in number of vesicles. Perhaps ATP utilization is low in the cold. If so, cooling decreases the rate of vesicle formation more than it slows vesicle disappearance. Rewarming may reverse this condition accounting for the accompanying increase in vesicle number.

In red blood cells, there is good evidence that ATP and not other nucleotides supply the necessary energy for transport processes (Hoffman, 1960; Garrahan and Glynn, 1967). Since red blood cells contain no mitochondria or enzymes for oxidative phosphorylation, ATP must come from glycolysis. In smooth muscle, both oxidative phosphorylation and glycolysis are present (Needham and Shoenberg, 1967; Daniel *et al.*, 1971d). Much work has been devoted to the source of ATP used by smooth muscle for contractility or transport phenomena. Uterine smooth muscle contains much less ATP than skeletal muscle (Walaas and Walaas, 1950a,b). The inability of smooth muscle to contract as many times as skeletal muscle after treatments with IAA and N_2 was attributed to differences in ATP content in the two muscles (Csapo and Gergely, 1950). Daniel and Robinson (1960) proposed that the main source of ATP for uterine smooth muscle ion movements was from glycolysis based on studies with several metabolic inhibitors. More recent evidence indicates that ATP from glycolysis can support both contractility and ion pumping in rat uterine smooth muscle. Inhibitors used to inhibit glycolysis, so that the utilization of ATP derived from oxidative phosphorylation could be studied, also inhibit contractility (Rangachari *et al.*, 1972; Daniel *et al.*, unpublished). In guinea pig papillary muscle, ATP produced by oxidative phosphorylation is believed to be used for both transport and contractility while glycolytically produced ATP is thought to be used preferentially for transport (Prasad and Macleod, 1969).

Results from this study showed that inhibition of both glycolysis and oxidative phosphorylation with IAA + DNP or IAA alone produced a depletion of both ATP content and the number of vesicles in the cells. The uncoupling of oxidative phosphorylation with DNP alone produced a decrease in ATP of only 35%, compared with the control, and then only after 6 hours treatment (Table 6). Similar changes in ATP contents of tissues were noted by Daniel and coworkers (1971d) after use of the above inhibitors. There were no changes in the number of cells with vesicles after DNP treatment although the vesicle number per cell was lower. These results suggest that glycolysis can maintain an appreciable ATP content in smooth muscle cells but indicate that glycolysis cannot maintain the initial level.

When glycolysis was inhibited with IAA and pyruvate was added as a substrate for oxidative metabolism, the ATP content of tissues was maintained at 10% of the control and vesicles were still present (Table 14). It is not known whether ATP was maintained at this low level due to a decrease in ATP in all cells or a complete depletion of ATP in 90% of the cells. Most likely some cells contained their normal complement of ATP as the vesicles in a few cells appeared near normal in structure. It is unlikely that a decrease of 90% of the ATP in all cells would result in near normal vesicles in some cells since this is approximately the threshold level of ATP necessary to maintain the vesicles or unless ATP from oxidative metabolism is used preferentially for vesicle maintenance only in such cells.

The failure of exogenously provided ATP to prevent vesicle destruction during metabolic inhibition (Table 14) probably indicates that ATP will not cross the cell membrane where it is needed for vesicle maintenance. In any case, ATP added outside cells is very rapidly hydrolyzed (see Allen and Daniel, 1964).

No evidence for a selective use of ATP from glycolysis or oxidative phosphorylation for the vesicular mechanisms was provided in the study described in this thesis.

In summary, the experiments described here were attempts to correlate the ATP content of tissues with the presence or absence of membrane vesicles in smooth muscle. When tissues were inhibited metabolically, a reduction in ATP content to 10% of the control levels was followed by a reduction in vesicle number and an increase in vesicle size. When ATP was rapidly depleted from the tissues a depletion of vesicles in the cells followed. These results are consistent with the postulate that membrane vesicles in uterine smooth muscle are maintained by ATP.

Sequence of changes. In the experiments with fresh myometrium, results of ATP, cation and water contents were similar to those reported by Daniel and Robinson (1971a,b,c) on studies made of whole uterine tissues. Metabolic inhibition with IAA + DNP or IAA caused ATP depletion, Na^+-gain, K^+-loss and swelling of tissues. Similar changes associated with similar ATP reduction occurred with ethacrynic acid treatment as observed by others (Daniel *et al.*, 1971d; Batra and Daniel, 1971).

Metabolic inhibition of fresh tissues with IAA + DNP caused the following sequence of events in uterine smooth muscle (Tables 2 and 3).

(a) Decreased ATP content (5 - 10 minutes).

(b) Increased number of cells with swollen vesicles 2.5 - 10 minutes).

(c) Decreased number of vesicles (10 minutes) and an increased vesicular size (5 minutes).

(d) Increased tissue Na^+ content (20 minutes) and a decreased K^+ content (10 minutes) along with a substantial increase in the number of cells without any observed vesicles.

(e) Increased H_2O content (30 minutes).

This sequence of events is consistent with the vesicular model proposed by Daniel and Robinson (1971c). When ATP is decreased rapidly by IAA + DNP, the Na^+ postulated to be bound within the vesicles at ATP-dependent sites would be released. Vesicle formation would also be decreased, if ATP is necessary for vesicle formation. There was a decrease in vesicle number following ATP reduction. The active extrusion of Na^+ by any mechanism would be prevented by ATP depletion which accounts for the increase in Na^+ content and decrease in K^+ content. After the loss of cellular K^+ for extracellular Na^+, probably because of inhibition of transport ATPase, the subsequent net gain in tissue water and in cellular Na^+ (with Cl^-) probably resulted from later inhibition of the vesicle pump.

Vesicle swelling. The possible explanations for apparent vesicular swelling with IAA + DNP are (a) a direct effect of the inhibitors on the vesicle membrane or on a contractile element within it to produce relaxation; (b) a coalescence of small vesicles to form larger vesicles; or (c) a distention of closed vesicles caused by the entry of water after the release of bound, osmotically inactive Na^+ within the vesicles secondary to a reduction in ATP.

Vesicular swelling did not occur with DNP treatment or consistently with IAA treatment. This indicated that neither DNP nor IAA alone had marked direct effects on vesicular structure and provided evidence against the first explanation above. If swelling of vesicles was linked to coalescence of vesicles (b above), then swelling should always be accompanied by reduced number of vesicles. This was not always true (i.e., IAA). Vesicular swelling could be related to the rapid decrease in ATP and release of Na^+ within the vesicles at a time when most of the vesicles are filled with Na^+ (c above). The Na released within the vesicles after IAA + DNP as a consequence of reduced ATP would cause water to move into the vesicles from the cytoplasm, increase the hydrostatic pressure within the vesicles and produce distention. In order for swelling to occur by this mechanism, the release of Na^+ must be more rapid than can be dissipated by diffusion and the vesicular membrane must be elastic.

If the increase in hydrostatic pressure is responsible for vesicular swelling, the vesicles must be closed or some restriction must be present at the neck of the open vesicle to prevent the Na^+ and water from flowing out of the vesicle to the extracellular fluid. There is no evidence for smooth muscle that membrane vesicles are ever closed completely and permanently from the extracellular space. Vesicles are often seen with no opening to the extracellular space but this probably means that the section is through the main body of the vesicle and does not include the connection to the exterior. Electron dense tracers, which are thought to penetrate the plasma membrane, have been used to study vesicles in smooth muscle. Lanthanum applied as a colloidal suspension during fixation (see Figure 4 , and Devine and Somlyo, 1970) and ferritin provided to fresh tissues before microscopy preparation (Somlyo *et al.*, 1971) have been found in vesicles, indicating communication channels to the extracellular space. A tracer such as ferritin, applied to living cells, could enter a vesicle whenever it is open to the extracellular space but lanthanum applied to fixed cells, would only enter vesicles with openings to the exterior. Thus, finding of ferritin within vesicles does not exclude that these vesicles may not have been closed at some stage of its existence. Such a finding with lanthanum has different implications. We do not know what fixation does to the vesicles. Perhaps fixation causes all vesicles to open and remain so. Freeze-etch techniques have also shown open vesicles but these too were demonstrated on glycerol-treated or fixed tissues (Devine *et al.*, 1971; Wolowyk, personal communication). It should be noted too, that in no study has evidence been presented that all vesicles contain markers.

Closed vesicles, similar in size and structure to those of smooth muscle, have been demonstrated in endothelial cells by serial sectioning (Bruns and Palade, 1968b). However, the validity of serial sectioning for the demonstration of closed vesicles is questionable since the vesicle size (1000 Å) is slightly greater than the section thickness (600 Å). Thus the vesicle neck which is much smaller (300 Å) may not be visible within the section. Therefore, the question of whether membrane vesicles are ever closed remains unanswered. Even if they are all open, diffusion delay in their necks could account for the swelling observed in IAA and DNP.

The changes which occurred in tissues inhibited with IAA alone were not as rapid as when DNP was also present. The absence of consistent vesicular swelling could be related to the slow decline in ATP. Perhaps the vesicles did not swell because Na^+ was released more slowly and leaked out of the vesicles through the necks or into the cytoplasm and did not accumulate in the vesicles. Another possibility is a selective action of IAA on membrane contractile function to open all the vesicles assuming that they are closed.

Ethacrynic acid, which inhibits glycolysis and oxidative phosphorylation in uterine smooth muscle in the concentrations used (Daniel *et al.*, 1971d), probably had effects similar to IAA. The vesicles were greatly increased in size and decreased in number after 4 and 6 hours treatment when ATP was absent in the tissues.

The apparent continuous swelling of vesicles after ATP depletion with IAA + DNP was probably due to different sensitivities of the cells. After 40 minutes treatment with IAA + DNP, there were only cells with swollen vesicles and cells with no vesicles (Table 3). With IAA, there was a small transient vesicular swelling (60 minutes) which may have been due to osmotic forces, elasticity and eventual equilibrium. The swelling which occurred later with IAA and ethacrynic acid may be due to their direct effect on the membrane.

Inhibition of transport ATPase. If formation of membrane vesicles in smooth muscle is dependent on the ouabain-insensitive pump (Daniel and Robinson, 1971c), treatment of tissues with ouabain should not affect vesicular structure. The results conformed to the predicted model. Ouabain had no effects on the number of membrane vesicles in fresh tissues. However, ouabain may have increased the activity of the volume pump as noted by decreased water content after treatment. The decreased water content was not caused by contraction produced by ouabain as the water loss was not blocked by adrenaline which inhibits ouabain-induced contraction (Daniel, 1964).

There were significant increases in vesicular number (2 hours) and size (6 hours) after ouabain treatment (Table 8). These changes may correspond to an increase in volume pump activity with ouabain. The large vesicles seen after ouabain treatment could be intracellular in origin and/or location but further work is necessary to define their nature. Perhaps these large vesicles are related to an increase in activity of the volume pump. The large vesicles could correspond to vacuoles seen in endothelial cells after ouabain treatment (Kaye *et al.*, 1965).

When ouabain was present in the rewarming solutions of Na$^+$-rich tissues, the tissues lost more weight than the controls and the vesicular population increased (Table 10). As in fresh tissues, ouabain may have increased the activity of the volume pump.

The stimulus for activation of the volume pump could be (a) a direct effect to activate some step in the volume pump (i.e., vesicle formation, discharge or both) or (b) inhibition of Na$^+$-K$^+$-ATPase stimulates the volume pump via some feedback system (i.e., more ATP or intracellular Na$^+$ is available for the volume pump when the Na$^+$-K$^+$-ATPase in inhibited).

Ouabain may act directly to stimulate both formation and discharge of the vesicles. This would account for the insignificant differences between the number of vesicles in the control and ouabain treated tissues at 6 hours. Another plausable model is that the stimulus for decreased volume is related to a feedback mechanism common to the volume pump and Na^+-K^+-ATPase. Evidence to support this reciprocal relationship comes from the observations that when the Na^+-K^+-ATPase was inhibited (rewarming tissues in ouabain or K^+-free solutions) there was a decreased water content accompanied by increased number of vesicles. When the Na^+-K^+-ATPase was allowed to function (rewarming of Na^+-rich tissues in normal Krebs), the water content decreased, but the vesicles did not change or decreased significantly in numbers. Possibly ATP availability at the membrane is responsible for this feedback phenomenon or it could be $[Na^+]_i$. When metabolically inhibited,fresh tissues could use ATP preferentially for the volume pump. This would explain Na^+ gain and K^+ loss occurring before H_2O gain during ATP reduction with metabolic inhibitors. Under conditions in which $[Na^+]_i$ was increased (Na^+-rich tissues) but when tissues were not inhibited metabolically, the ATP could be used preferentially by Na^+-K^+-ATPase. This would explain why no changes or decreased vesicles were found during rewarming of Na^+-rich tissues under conditions of maximal Na^+-K^+-ATPase activity (rewarming in normal Krebs).

Evidence for a Ca^{++}-dependent mechanism. In part of this study, Rangachari's (1972) results with Na^+-rich tissues were confirmed. Tissues made Na^+-rich by cooling and then rewarmed in K^+-free solution lost weight. The loss of weight from rewarmed Na^+-rich tissues was prevented by the presence of IAAmide or with Ca^{++}-free solutions. When tissues were treated with IAAmide during rewarming, in the same concentrations and under identical conditions to those Rangachari (1972) found did not affect the ATP but inhibited water loss, the vesicles were significantly increased in number as were the controls which lost water. IAAmide did not affect the formation of vesicles possibly because the ATP was not diminished. Perhaps IAAmide acts directly on a vesicular element to inhibit discharge. Inhibition of discharge may then inhibit formation of vesicles by some feedback system.

The presence of Ca^{++} was necessary for the weight loss and increase in vesicular number during rewarming in K^+-free solutions. Possibly a Ca^{++}-dependent ATPase located around the vesicles is responsible for the operation of the volume pump as suggested by Rangachari (1972). A Ca^{++}-dependent ATPase responsible for cell volume has also been proposed for kidney cells (Rorive and Kleinzeller, 1972).

Ouabain and rewarming Na^+-rich tissues in K^+free solutions results in contracture of the tissue (Daniel, 1964; Rangachari *et al.*, 1972). One possible model ruled out by other studies was that the water loss observed with ouabain and during rewarming was due to a loss of water from the extracellular space caused by contracture of the tissue rather than a loss of isotonic solution from the cells caused by a mechanochemical system. The ouabain induced contracture can be prevented by adrenaline (Daniel, 1964) but the water losses were not inhibited. Also, rewarming Na^+-rich tissues in normal Krebs-Ringer results in hyperpolarization and relaxation (Taylor *et al.*, 1970) but the tissues still lost water under these conditions.

Non-specificity. K^+ was able to substitute in place of Na^+ in the water loss and stimulating the increase in vesicle number during rewarming. Solutions with high K^+ concentrations could inhibit Na^+-K^+-ATPase in rat myometrium as observed for other tissues (Skou, 1965). Thus, like ouabain and K^+-free solutions, whenever the Na^+-K^+-ATPase was inhibited both the volume pump activity and the number of vesicles increased and in this case stimulated by K^+.

Li^+, on the other hand, will not substitute for Na^+ in the Na^+-K^+-ATPase enzyme reaction (Skou, 1965) and may not substitute for Na^+ in stimulation of volume pump. This accounts for the lack of increased vesicles and lack of water loss in Li^+-rich tissues rewarmed in Li^+ solutions.

2. *Vesicle Structure, Size and Distribution*

Both the plasma and vesicular membrane of uterine smooth muscle are composed of three layers approximately 80 Å thick. This estimate corresponds to measurements of smooth muscle plasma membrane of rat vas deferens (70 - 110 Å) reported by Burnstock (1970). Earlier estimates of 150 - 250 Å thickness (Mark, 1956; Bergman, 1958) could be due to technical difficulties such as poor resolution with the electron microscope or earlier methods of tissue preparation.

The vesicles are almost spherical in uterine smooth muscle with average diameters of about 1000 Å. The vesicle size in vascular and intestinal smooth muscle has been estimated at 700 to 2500 Å (Caesar *et al.*, 1957; Prosser *et al.*, 1960; Rhodin, 1962; Simpson and Devine, 1966). This range of sizes could be related to the various methods of fixation used, since permanganate fixation results in smaller vesicles than glutaraldehyde or osmium fixation (Yamauchi and Burnstock, 1969).

Two sizes of vesicles were found in several types of smooth muscle studied by Prosser and coworkers (1960). They noted that the larger vesicles (0.1 - 0.3 μ) were usually beneath areas of smaller surface vesicles and that a mixture of large and small vesicles formed longitudinal rows down the muscle cell. Fifteen to twenty rows of vesicles per cell were estimated by the above workers based on distances between vesicles and the cell diameters. Longitudinal rows of vesicles in smooth muscle cells have repeatedly been found but the significance of this arrangement is unknown (Simpson and Devine, 1966; Gabella, 1971; Wells and Wolowyk, 1971). Perhaps the organization of vesicles into rows is related to the longitudinal orientation of (1) mitochondria and/or endoplasmic reticulum or (2) the attachment sites of filaments. A close association between vesicles and mitochondria (Wolowyk, personal communication) or endoplasmic reticulum (Gabella, 1971, 1972; Somlyo *et al.*, 1971) has been described for smooth muscle. On the other hand, vesicles appear in membrane areas not occupied by the dense areas which are thought by some to be regions of myofilament attachment (Lane, 1965; Nemetschek-Gansler, 1967).

The smooth muscle cells examined in this quantitative study were all cut in cross-section so that the chance of bias was removed. Control tissues sectioned in this manner contained approximately 1000 vesicles per millimeter of membrane. This number of vesicles was very consistent in control tissues and it did not vary much even after incubation of tissues up to 6 hours in Krebs-Ringer solution. This indicated that the method used for vesicle measurements was reliable.

The vesicular membrane has been estimated to increase the area of the plasma membrane of mouse intestinal smooth muscle by 25% based on surveys of large numbers of prints of longitudinal and cross-sectioned cells (Rhodin, 1962). Similar measurements of guinea-pig taenia coli have yielded an estimated increase in membrane area as high as 70% (Goodford, 1970). The reason the two estimates disagree could be due to the species differences or differences in methods of fixation. Vesicles are thought to vary in number because of tissue and species differences (Rhodin, 1962), maturity of cells and between fixatives used (Yamauchi and Burnstock, 1969).

Taking the average number of vesicles along the membrane of control tissues (ca. 1000 vesicles/mm or 1 vesicle/μ, Table 2), the estimated vesicular density on the surface of smooth muscle cells would be 10 vesicles/μ^2. With this population of vesicles and the surface area of a 1000 Å vesicle (0.0314 μ^2), one can compute that the vesicles would increase the membrane area by 31.4%. This calculation is remarkably close to the calculation for intestinal smooth muscle (25%, Rhodin, 1962).

Perhaps Na^+-K^+-activated ATPase enzymes are located on the vesicular membrane. Repeating units, outlined by electron dense lanthanum, were found only along the vesicular membranes. Similar treatment of chloride cells of marine animals revealed similar particles associated with vesicles and tubules which were believed to function in electrolyte transport (Ritch and Philpott, 1969). More particles within the vesicular membrane were observed in freeze-etch studies of guinea-pig taenia coli (Wells and Wolowyk, 1971). Perhaps Na^+-K^+-ATPase enzymes are preferentially located or optimally active on the curved vesicular membrane.

3. *Rate of Vesicular Pumping*

Daniel and Robinson (1970) calculated that the Na^+ efflux from cells of the fast cellular fraction (vesicular) in fresh tissues was approximately 5.9 pmoles Na^+ cm^{-2} sec^{-1}. Taking the concentration of Na^+ each vesicle is capable of accumulating (900 mM, based on 920 Å diameter vesicles as calculated by Goodford *et al.*, 1968), and the population of vesicles per surface area of the cell (10 vesicles/u^2 or 1 x 10^9 vesicles/cm^2, as estimated in this study), one can calculate that only 1.3% of the vesicles (12.8 x 10^6 vesicles cm^{-2} sec^{-1}) need release their contents every second to achieve the measured efflux. If 1.3% of the vesicles are discharged and formed every second, the time for 50% of the vesicles to turnover would be approximately 38 seconds. This seems like a long time in respect to the lifetime of vesicles in other cells (1 second in endothelial cells, Shea and Karnovsky, 1969) but perhaps only a small percentage of vesicular binding sites are occupied by cellular Na^+. It would be more disturbing if there were not enough vesicles to account for the loss of Na^+.

4. *Summary*

This quantitative ultrastructural study was designed to test the vesicular hypothesis. The data presented in this study provides evidence of good correlation between characteristics of the volume pump in uterine smooth muscle, as described by others (Daniel and

Robinson, 1971a,b,c; Rangachari, 1972; Rangachari *et al.*, 1972), and characteristics of membrane vesicles. Both systems (a) are ATP-dependent; (b) do not require external K^+ and are not inhibited by ouabain; (c) are Ca^{++}-dependent and may involve a contractile element; (d) lack specificity for Na^+. Therefore, it is reasonable to suggest that the membrane vesicles are sites of volume control in uterine smooth muscle.

CHAPTER 3

EVALUATION OF THE PYROANTIMONATE TECHNIQUE FOR LOCALIZATION OF TISSUE SODIUM

CHAPTER 3

EVALUATION OF THE PYROANTIMONATE TECHNIQUE FOR LOCALIZATION OF TISSUE SODIUM

A. Objectives

Potassium pyroantimonate has been widely used to localize tissue sodium for electron microscopy by the formation of electron dense precipitates with sodium. The validity of this technique has been questioned (Bulger, 1969; Clark and Ackerman, 1971; Sumi, 1971; Sumi and Swanson, 1971). The object of this investigation was to evaluate the technique because of its possible usefulness in localizing sites of Na^+ transport in smooth muscle (see chapter 2 in this thesis).

To accurately localize sodium pyroantimonate must precipitate sodium quantitatively. If pyroantimonate precipitates cellular sodium quantitatively, tissues fixed in the presence of pyroantimonate should contain as much sodium as originally present. Furthermore, the amount of precipitate seen in the tissues by electron microscopy should be directly related to the sodium content after alteration of its level. To test whether pyroantimonate precipitates tissue sodium quantitatively, tissues with normal and altered sodium contents were fixed with and without pyroantimonate for electron microscopy. Some fixed tissues were analyzed, for sodium and other cations which also precipitate with pyroantimonate, after each step in the preparation of the tissues for the electron microscope. Other tissues were examined in the electron microscope for the appearance of deposits.

B. Materials and Methods

1. *Tissues*

Pieces of uterine tissue used in this study were treated as described in Chapter 2 of this thesis.

Fresh tissues prepared for ^{22}Na analysis were incubated in aerated ^{22}Na Krebs-Ringer medium for two hours. Sodium-rich tissues were prepared by incubation in a potassium-free ^{22}Na Krebs-Ringer solution overnight at 5°C. The specific activities of these solutions were approximately 1.77×10^6 cpm/mmole Na (ca. 2.5×10^5 cpm/ml).

2. *Solutions*

The Krebs-Ringer solutions used in this study were identical to the solutions described previously (Chapter 2, Table 1). ^{22}Na Krebs-Ringer and potassium-free ^{22}Na Krebs-Ringer solutions were prepared by adding trace amounts of ^{22}NaCl (1.77×10^6 cpm/mmole Na) obtained from Nuclear Science and Engineering Corporation, Pittsburgh, Pennsylvania.

Two percent potassium pyroantimonate (40 mM; as $K_2H_2Sb_2O_7.4H_2O$;Fisher Scientific) was prepared by boiling the salt in distilled water until it dissolved, and readjusting the volume. The pH of this solution was lowered to 7.4 with a few drops of 0.1 N HCl. ^{124}Sb-labelled potassium pyroantimonate ($K_2H_2{}^{124}Sb_2O_7.4H_2O$) was obtained from New England Nuclear Corporation, Boston, Massachusets. Solutions containing 20 µci ^{124}Sb-pyroantimonate/100 ml were prepared by adding

5 ml of the labelled 2% solution to 95 ml of the unlabelled 2% solution.

OsO_4 fixative was prepared by dissolving 0.1 gram in 10 ml of either distilled water (pH 7.0, ca. 40 mOsm) or 2% potassium pyroantimonate solution (pH 7.4, ca. 170 mOsm). 3% glutaraldehyde (pH 7.0, 360 mOsm) was made neutral by removing the acidic byproducts with charcoal or by raising the pH with a few drops of 0.01 N NaOH. 3% glutaraldehyde with pyroantimonate (pH 7.4, 450 mOsm) was prepared by diluting the acidic 70% concentrated glutaraldehyde (Ladd Research Industries) with the basic 2% saturated pyroantimonate solution. All fixatives were made just prior to use. Osmolarities of solutions were measured with an Advanced Instruments osmometer.

All solutions were analyzed spectrophotometrically for their cation constituents. The specific activities for radioactive solutions (cpm/mmoles) were calculated from their cation concentration and the cpm per unit volume.

3. *Fixation and Dehydration*

After incubation, one horn from each uterus was trimmed at both ends, cut into 5 pieces approximately 5 x 5 x 0.5 mm, blotted on paper, weighed (< 50 mg wet weight) and 4 pieces transferred to test tubes containing 1 ml of fixative; the remaining piece was used as the unfixed control for analysis. Four pieces from the opposide horn were fixed in the presence of pyroantimonate; the remaining piece was prepared and embedded for electron microscopy.[1] Some tissues were fixed at room temperature for 1 hour in either 1% OsO_4 alone or 1% OsO_4 plus 2% potassium pyroantimonate. Other tissues were fixed in 3% glutaraldehyde at room temperature for 3 hours with and without pyroantimonate, followed by post-fixation in 1% OsO_4. The tissues were then washed once in 1 ml of distilled water for 15 minutes. Dehydration was accomplished in 1 ml each of a graded series of alcohols for 15 minutes at each step. Between each step the tissues for analysis were rapidly dipped in water to remove adherent solution and the water was added back to the previous solution for analysis.

4. *Electron Microscopy*

All tissues prepared for electron microscopy were embedded in Epon 812. Silver to grey sections were cut on a Porter-Blum ultramicrotome and placed on bare 300-mesh grids. Sections were examined without stain in a JEM-7A electron microscope.

5. *Tissue Cation Analysis*

After each step of fixation and dehydration samples were removed, rinsed, dried overnight at 105°C to a constant weight (< 10 mg dry weight), and digested in concentrated nitric acid and 30% hydrogen peroxide (0.1 ml each) on a hot sand bath. The resulting solution was then diluted to 25 ml with distilled water for analysis, as were the fixation and dehydration solutions. Solutions were analyzed for sodium and potassium in an EEL Flame Photometer, and for calcium and magnesium by atomic absorption in a Unicam SP-90A against standard solutions. All glassware was washed in dilute EDTA and nitric acid solutions prior to use. For calcium analysis 0.35 mmoles of lanthanum chloride was added to samples and standards to release calcium from any tissue phosphate. Appropriate blanks for all solutions were prepared and subtracted from the readings of tissue and solutions.

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[1] Similar results were obtained if the tissues from several animals were divided before incubation and taken randomly for fixation and control (Figures 20, 21 and 22).

To estimate intracellular sodium, the extracellular sodium content of tissues was first estimated by multiplying the previously determined fractional volume of the extracellular space (0.46 as measured with inulin; Daniel, 1963a) by the sodium concentration in the incubation solution (= extracellular sodium concentration). The extracellular sodium content based on fresh weight was converted to a dry weight basis by dividing by the ratio of final dry weight to fresh weight. The intracellular sodium content was obtained by subtracting the extracellular sodium content from the total tissue sodium content. The dry/wet weight ratios obtained for tissues before fixation were approximately 20% (see Table 16).

6. *Isotope Analysis*

^{22}Na and ^{124}Sb-labelled samples were counted in a well-type scintillation counter (Picker X-ray Engineering Limited, Canada) by changing the settings to correspond to the counting peak of the isotopes. ^{22}Na and ^{124}Sb were counted at 425 and 500 lower level settings respectively, with a window width of 100 and high voltage set at the counting plateau (1050 volts). Tissues were counted for ^{22}Na and later the control tissues were analyzed spectrophotometrically to determine the percent of Na^+- ^{22}Na exchange. Uptakes of labelled sodium and pyroantimonate in tissues were estimated by dividing the counts per minute per kilogram of tissue weight by the respective specific activities of the incubation solution.

C. Results

1. *Effect of Pyroantimonate on Cation Determination*

Before cation contents could be measured in pyroantimonate treated tissue, it was necessary to show that pyroantimonate does not interfere with the analyses. To reproduce the conditions for tissue sodium analysis, 2 ml of 2% potassium pyroantimonate (40 mM) was added to 1 ml of various concentrations of sodium chloride (5 - 80 mM). A precipitate formed at all concentrations, but disappeared on dilution to 25 ml, except in those solutions above 0.8 mM NaCl (final concentrations). The diluted standards were then analyzed by flame photometry 60 minutes later. The two curves, of solutions with and without pyroantimonate, deviated near the point where precipitate remained after dilution (Figure 16a). These results indicate that pyroantimonate interfered with the determinations only at sodium concentrations above this point. The presence of OsO_4

of glutaraldehyde fixatives did not alter these curves.

Because pyroantimonate also precipitates with calcium and magnesium and because tissues were to be analyzed for these cations, the effect of pyroantimonate on analyses of these cations was studied. Figure 16b shows the effect on calcium analysis. When 2 ml of 2% pyroantimonate solution was added to 1 ml of calcium standards (62.5-1250 µM $CaCl_2$) precipitates were formed at all $CaCl_2$ concentrations above 750 µM and these solutions remained turbid following dilution to 25 ml. The addition of $LaCl_2$ (0.33 mmoles) to all solutions after dilution resulted in a second precipitate, when pyroantimonate was present. When the solutions were analyzed, the point of divergence (solid *vs.* dotted line) was below the point of precipitate formation with $CaCl_2$ prior to dilution and visible turbidity after dilution. Since lanthanum was added to displace calcium from any phosphate present in tissue, it was crucial to determine if the precipitation of lanthanum by pyroantimonate prevented this effect. To test this, an amount of phosphate equal to that of lanthanum and greater than that in the tissue samples was added after dilution to the calcium standards containing pyroantimonate; this only slightly decreased calcium measurements at higher calcium concentrations suggesting that sufficient lanthanum was still present to release calcium ions (lower curve, dashed line).

The sodium and calcium concentrations at which pyroantimonate reduced spectrophotometric readings were above 0.8 mM NaCl and 10 µM $CaCl_2$.

Pyroantimonate did not reduce the atomic absorption readings of diluted magnesium solutions which included the expected concentra-

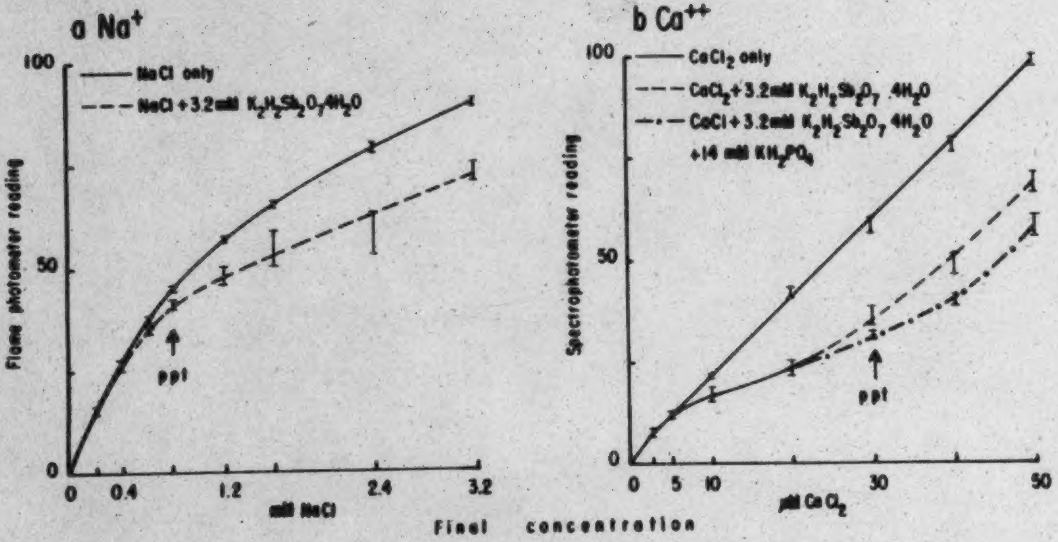


Figure 16. Effect of pyroantimonate on Na^+ and Ca^{++} determination.
(a) Na^+ determination by flame photometry. Two ml of 2% potassium pyroantimonate (40 mM $K_2H_2Sb_2O_7.4H_2O$) was added to 1 ml of various concentrations of sodium chloride solution and diluted to 25 ml. The top curve shows the flame photometer readings *vs.* the final concentration of sodium chloride solutions only (solid line); the bottome curve shows sodium chloride plus the added pyroantimonate (dashed line). The two curves deviated at the point (marked ppt) where precipitate remained after dilution. Each bar represents the range of four determinations.

(b) Ca^{++} determination by atomic absorption. The curves show the atomic absorption readings *vs.* the final concentration of calcium chloride standards without pyroantimonate (top, solid line) and with added pyroantimonate (middle, dashed line). The bottom curve (dash-dotted line) shows the effect of the addition of phosphate (KH_2PO_4) to pyroantimonate-containing solutions, in a final concentration equal to that of lanthanum (14 mM). Each bar represents the range of four determinations.

tion of tissue magnesium.

2. *Effect of Fixation and Dehydration on Na^+ and K^+ Content of Fresh Tissue*

OsO_4. The effect of fixation and dehydration on sodium content of fresh tissue fixed in OsO_4, with and without pyroantimonate, is shown in Figure 17a. Only about one-fourth of the original sodium remained in tissue fixed in OsO_4 alone, and this amount was close to the estimated intracellular sodium content. Less sodium remained in tissue fixed in the presence of pyroantimonate (ca. 1/10 of the original amount). In each case, the sodium lost from tissues was accounted for in the fixative solution. Once the tissues were fixed, little sodium was lost into the dehydrating solutions.

Approximately one-fourth of the original tissue potassium remained after fixation of OsO_4 (Figure 17b). Tissues fixed in the presence of potassium pyroantimonate had a high potassium content because of the added potassium salt. Like sodium, little or no potassium was lost to the dehydration solutions once the tissues were fixed.

Glutaraldehyde. Less sodium remained in the tissues than after OsO_4 (ca. 1/10 *vs.* 1/4), and the presence of pyroantimonate had little effect (Figure 17c). Tissues fixed in glutaraldehyde without potassium pyroantimonate retained only negligible amounts of potassium (Figure 17d). Thus, both more sodium and more potassium were lost from tissues fixed in glutaraldehyde than from tissues fixed in OsO_4. When potassium pyroantimonate was present, the potassium content of glutaraldehyde-

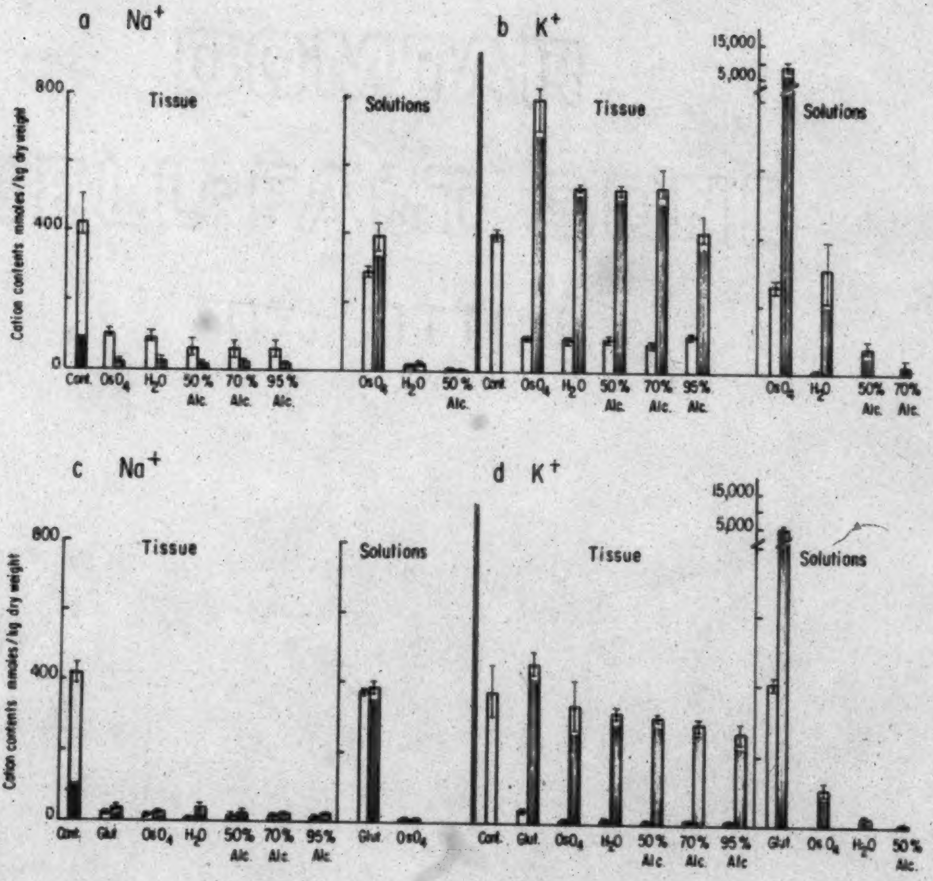
Figure 17. Effect of fixation and dehydration on Na^+ and K^+ contents of fresh tissue. The bar graphs illustrate the Na^+ and K^+ content of rat uterine tissues before and after each step of fixation (in 1% OsO_4 alone or 3% glutaraldehyde followed by OsO_4) and dehydration. The bars at the extreme left of each subfigure (marked cont) indicate the amount of cations in uteri before fixation, but after 30 minutes incubation in Krebs-Ringer solution. The shaded part of these bars shows the estimated intracellular content of Na^+. The clear bars indicate the amount of cations remaining in the tissue at that step, or contained in the corresponding solution when pyroantimonate was absent from the fixative; the cross-hatched bars when pyroantimonate was present in the primary fixative. Solutions containing no electrolytes were omitted. The height of each bar except the control is the average of 4 determinations (see Methods); the range is also indicated.

(a) Na^+ content of tissues before and after OsO_4 fixation and dehydration, and of corresponding solutions.

(b) K^+ content of corresponding tissues and solutions.

(c) Na^+ content; glutaraldehyde fixation.

(d) K^+ content; glutaraldehyde fixation.


a Na+
Tissue
Solutions
800
400
0
Cation contents mmoles/kg dry weight
Cont.
OsO4
H2O
50% Alc.
70% Alc.
95% Alc.
OsO4
H2O
50% Alc.
b K+
Tissue
Solutions
15,000
5,000
Cont.
OsO4
H2O
50% Alc.
70% Alc.
95% Alc.
OsO4
H2O
50% Alc.
70% Alc.
c Na+
Tissue
Solutions
800
400
0
Cation contents mmoles/kg dry weight
Cont.
Glut.
OsO4
H2O
50% Alc.
70% Alc.
95% Alc.
Glut.
OsO4
d K+
Tissue
Solutions
15,000
5,000
Cont.
Glut.
OsO4
H2O
50% Alc.
70% Alc.
95% Alc.
Glut.
OsO4
H2O
50% Alc.

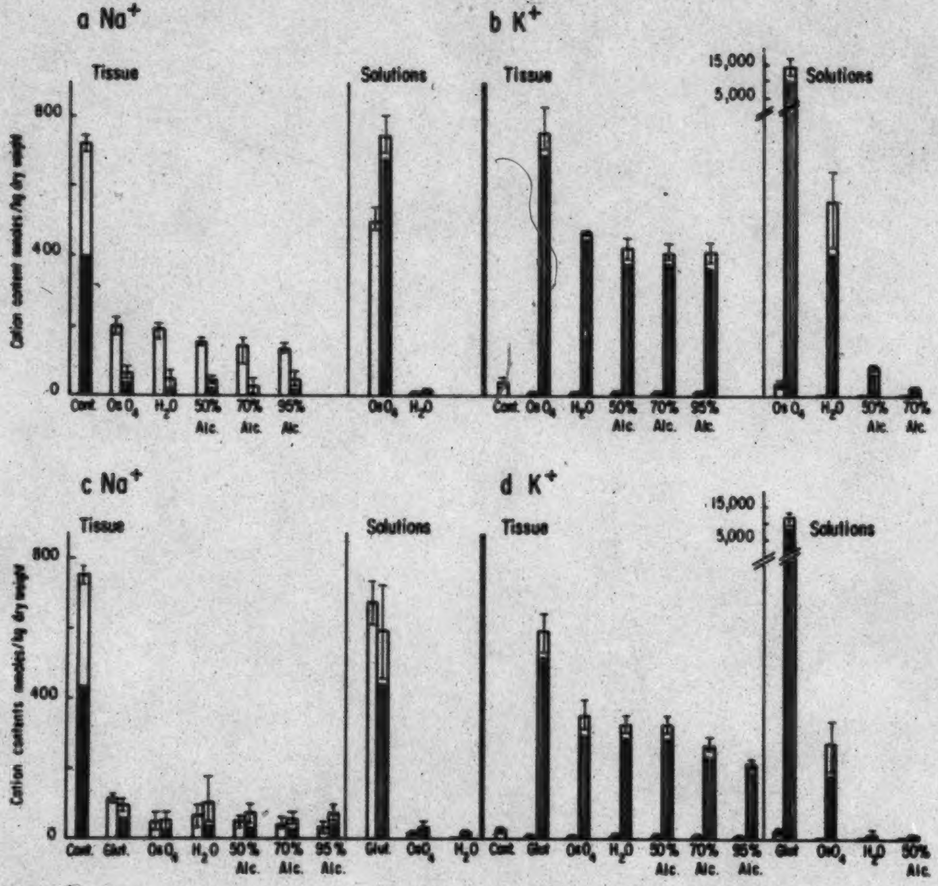
fixed tissues was increased, but was slightly lower than that of OsO_4-fixed tissues.

3. *Effect of Fixation and Dehydration on Na^+ and K^+ Contents of Tissues with Altered Cation Content*

Tissues with elevated or decreased sodium or potassium contents prior to fixation followed the same general pattern as fresh tissues during fixation and dehydration, but the cation levels throughout the procedures were generally altered in the same direction as initially (Figures 18 and 19).

4. *^{22}Na Content of Fixed Tissue*

As a check on the validity of the spectrophotometric determinations, tissues were labelled with ^{22}Na. ^{22}Na-Na^+ exchange was almost complete in both fresh tissues (ca. 95%) and sodium-rich tissues (ca. 98%) as previously found by Daniel and Robinson (1970). More ^{22}Na was retained by fresh and sodium-rich tissue fixed in OsO_4 than tissues fixed in glutaraldehyde (Figure 20). The presence of pyroantimonate in the fixative decreased the ^{22}Na content of OsO_4-fixed tissues and had little effect on the low ^{22}Na content of glutaraldehyde-fixed tissues. These results confirm those obtained by tissue sodium analysis.



<ins>Figure 18</ins>. Na^+ and K^+ contents of tissue made sodium-rich by incubation overnight at 5°C in potassium-free Krebs-Ringer solution. Results are indicated as in Figure 17.

(a) Na^+ content; OsO_4 fixation.

(b) K^+ content; OsO_4 fixation.

(c) Na^+ content; glutaraldehyde fixation.

(d) K^+ content; glutaraldehyde fixation.

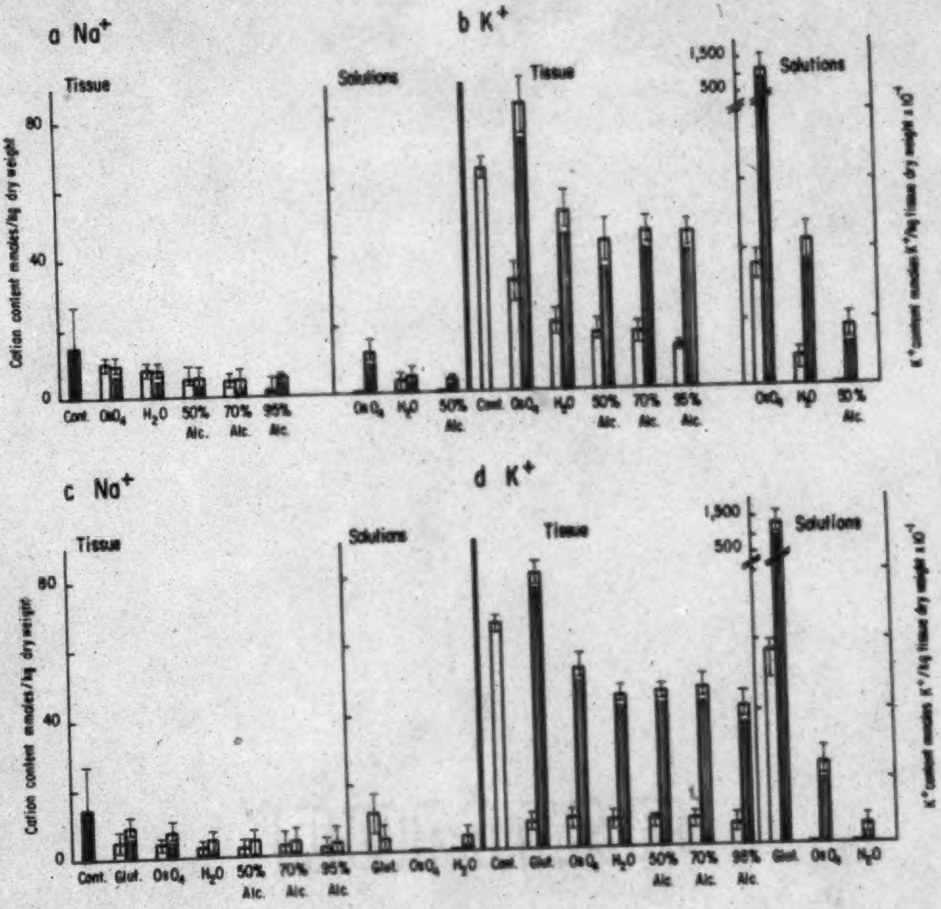


Figure 19. Na$^+$ and K$^+$ contents of tissue made sodium-poor by incubation for 1 hour in sodium-free Krebs-Ringer solution. Results indicated as in Figure 17.

(a) Na$^+$ content; OsO$_4$ fixation.

(b) K$^+$ content; OsO$_4$ fixation.

(c) Na$^+$ content; glutaraldehyde fixation.

(d) K$^+$ content; glutaraldehyde fixation.

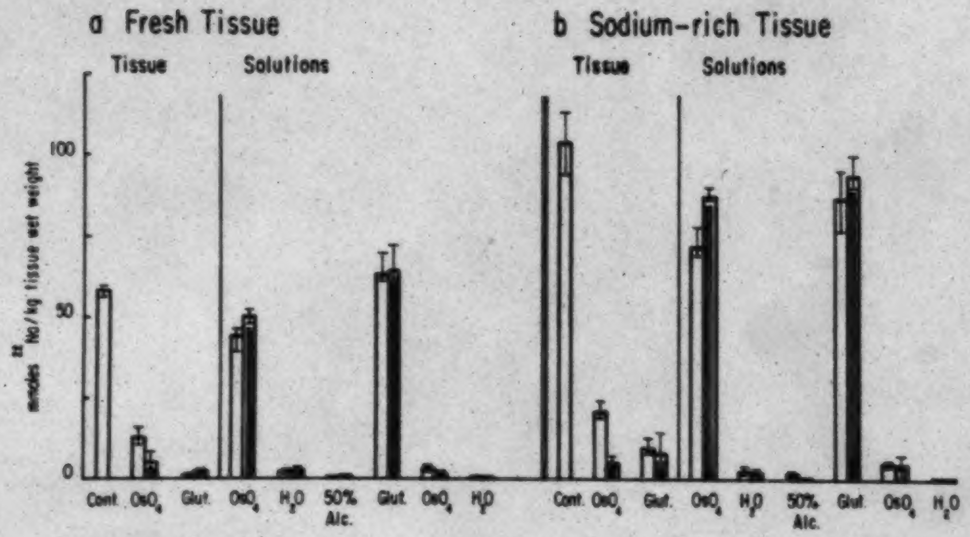


Figure 20. ^{22}Na content of tissues before and after fixation and dehydration. Results indicated as in Figure 17.

(a) ^{22}Na content of tissue incubated for 2 hours in ^{22}Na-Krebs-Ringer solution (fresh tissue); fixation in OsO_4 or glutaraldehyde followed by OsO_4.

(b) ^{22}Na content of tissue incubated overnight in K^+-Krebs-Ringer solution (^{22}Na-rich); fixation in OsO_4 alone or glutaraldehyde followed by OsO_4.

5. *Pyroantimonate Content of Fixed Tissue*

Figure 21 shows the contents of ^{124}Sb-pyroantimonate and also ^{22}Na in fresh, sodium-rich and sodium-poor tissues after OsO_4 and glutaraldehyde fixation.[2] Tissues fixed in OsO_4 contained 3 to 4 times more ^{124}Sb-pyroantimonate than glutaraldehyde-fixed tissues. ^{124}Sb-pyroantimonate content was not increased in sodium-rich tissues or decreased in sodium-poor ones. Thus, there was no relationship between the sodium content and ^{124}Sb-pyroantimonate content after fixation.

6. *Effect of Fixation and Dehydration on Ca^{++} and Mg^{++} Contents of Fresh Tissues*

Little calcium or magnesium was lost from tissues when OsO_4 or glutaraldehyde was used without pyroantimonate (Figure 22, clear bars). When pyroantimonate was added to OsO_4, less of these two divalent cations remained in the tissue; when added to glutaraldehyde there was much less effect (Figure 22, hatched bars). There was much more calcium than magnesium loss with both fixatives though each

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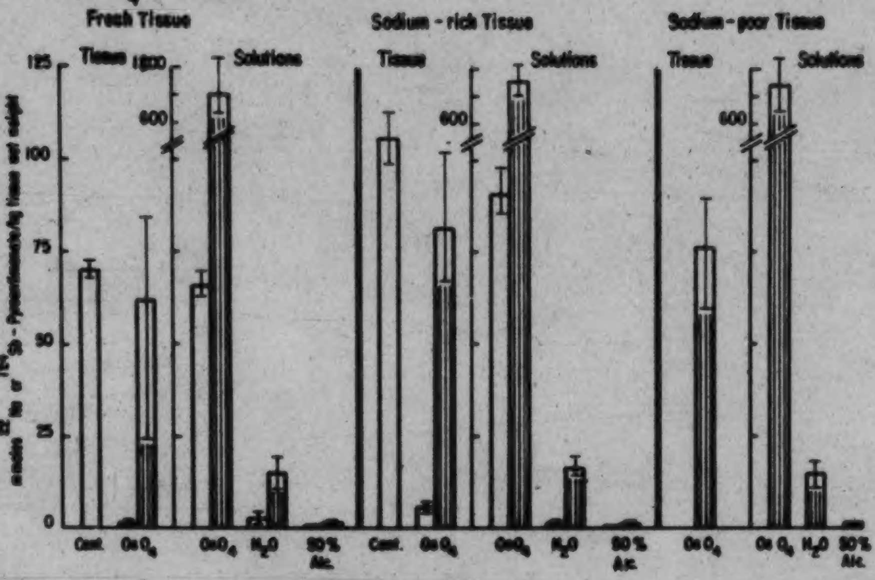
[2] The results shown are from two separate experiments, each with tissues containing only one isotopic label, either ^{22}Na exchanged before fixation or ^{124}Sb provided during fixation. Other tissues were labelled with both isotopes by preloading with ^{22}Na and fixing in the presence of ^{124}Sb-pyroantimonate and counted simultaneously. Results similar to those with the single label were obtained but greater variation occurred due to the narrow window setting required to separate the two isotope peaks.

Figure 21. ^{124}Sb-pyroantimonate and ^{22}Na content of fixed tissues. The clear bars show the content of ^{22}Na in fresh and sodium-rich tissues before and after fixation and dehydration in OsO_4 or glutaraldehyde. The cross-hatched bars show the content of ^{124}Sb-pyroantimonate which remained in the tissues when fixed in its presence. Results are from two experiments. The height of each bar is the average of 8 values.

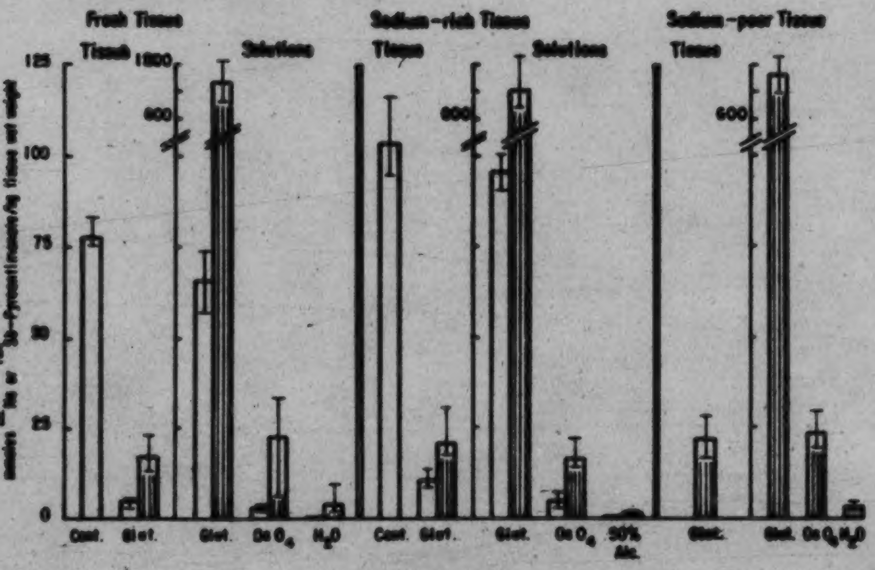
(a) OsO_4 fixation of fresh, sodium-rich and sodium-poor tissue.

(b) Glutaraldehyde fixation of the same three types of tissue.

a OsO_4 Fixation



b Glutaraldehyde Fixation



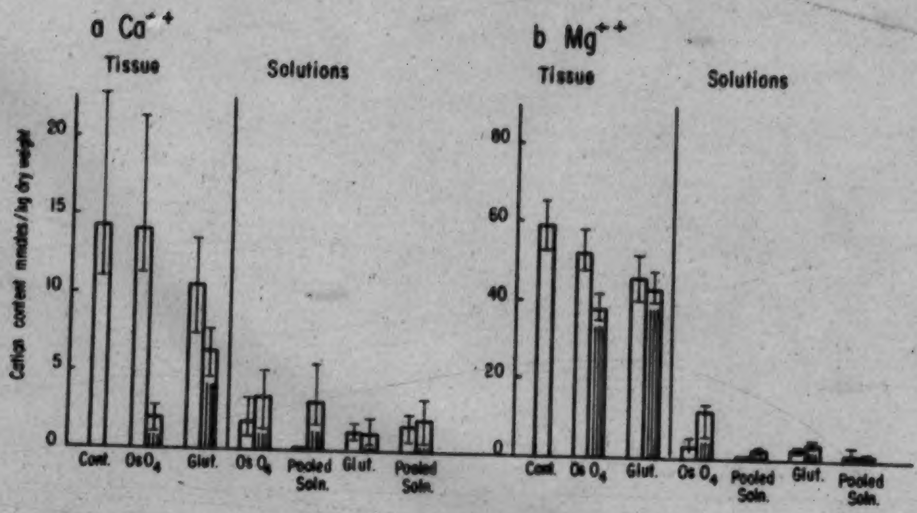


Figure 22. Ca^{++} and Mg^{++} contents of fresh tissues before and after fixation and dehydration. Each bar is the average of 8 values with the range. All solutions used after fixation were pooled and analyzed together.

Results are indicated as in Figure 17.

(a) Ca^{++} content; fixation in OsO_4 or glutaraldehyde followed by OsO_4.

(b) Mg^{++} content; fixation in OsO_4 or glutaraldehyde followed by OsO_4.

loss was more pronounced with OsO_4. As with sodium, the additional loss of cation caused by pyroantimonate was greater when the loss in the fixative alone was less.

7. *Effects of Other Solutions on Sodium Loss During Fixation*

Two possible explanations for the reduction in sodium content of tissues fixed with OsO_4 in the presence of pyroantimonate are, (1) that external pyroantimonate complexes and removes the sodium from the tissue, or (2) that tissue sodium exchanges for potassium present with the pyroantimonate. When the same concentration of the potassium salt of another impermeable anion, sulfate, which forms a more soluble sodium salt, was added to OsO_4, the tissue sodium content was reduced like that of pyroantimonate-treated tissue (Table 15). The values in Table 15 are lower than those shown in Figure 17, probably due to the low initial quantity of sodium in the tissues (ca. 340 *vs.* 400 mmoles Na^+/kg dry weight). The same effect of lower sodium content was also seen when tissues were incubated in high potassium solutions with permable anions (KCl Krebs-Ringer solution, Figure 19). These results suggest that potassium exchanges for tissue sodium rather than pyroantimonate complexing it from the tissues.

Tissues incubated in distilled water for the same length of time contained the same amount of sodium as those fixed in OsO_4. Tissues incubated in distilled water were expected to contain less sodium due to swelling and disruption of cells. Tissues placed in isosmolar sucrose solution (280 mOsm), in which swelling would not be expected to occur and which had no exchangeable cations, contained less sodium than OsO_4-fixed tissues or tissues incubated in distilled water (Table 15). These were unexpected findings for which I have no apparent explanation.

Tissues fixed in glutaraldehyde, like those incubated in iso-osmolar sucrose for an equal time, contained much less sodium after fixation than OsO_4-fixed tissues. The addition of potassium pyroantimonate had little effect (Table 15).

8. EM Observations

The distribution of pyroantimonate precipitates seen in tissues fixed in OsO_4 (Figure 23a,c,e) was different from precipitates observed in glutaraldehyde-fixed tissues (Figure 23b,d,f). OsO_4-fixed tissues contained fine precipitates in the nucleus associated with the heterochromatin, in the nucleolus, cytoplasm, mitochondria, and extracellular space. Tissues fixed in glutaraldehyde contained larger precipitates and these were observed in only a few cells which had swollen endoplasmic reticulum and a swollen nuclear envelope. In cells with intracellular precipitates, these were found in the interchromatin area of the nucleus, in the nucleolus, mitochondria, and extracellular space. With either fixative, precipitates were neither increased nor decreased in sodium-rich or sodium-poor tissue (Figure 23c-f).

TABLE 15

Effects of various solutions on Na^+ content of fresh tissue

Fixative	With Fixative	Without Fixative
1% OsO_4	OsO_4 64 (44-76)	Water 58 (12-89)
	OsO_4 + K-Ant.* 15 (11-16)	Water + K-Ant.* 17 (14-20)
	OsO_4 + K_2SO_4 7 (4-10)	Water + K_2SO_4 18 (13-27)
	OsO_4 51 (32-72)	Sucrose 10 (6-15)
3% Glutaraldehyde	Glutaraldehyde 15 (11-18)	Sucrose 15 (8-28)

Values shown are the average sodium content (mmoles Na^+/kg dry wt.) of 4 fresh tissues (with range in brackets) after fixation or incubation in the solution indicated.

*Potassium pyroantimonate.

Figure 23. Effect of fixative on location of pyroantimonate deposits in rat uterine smooth muscle. Electron micrographs of unstained tissue; *N*, nucleus; *n*, nucleolus; *M*, mitochondria; *E*, extracellular space occupied by collagen. Magnification x 12,000.

(a,b) *Fresh tissue.*

(a) Tissue fixed in 1% OsO_4. Small, fine precipitates can be seen in the nucleus where they appear associated with the chromatin material; in mitochondria, cytoplasm, extracellular space, and in the nucleolus.

(b) Tissue fixed in 3% glutaraldehyde and postfixed in 1% OsO_4. Coarse precipitates were found within only a few cells and were located in the interchromatin region of the nucleus, on the nucleolus and in mitochondria. Two cells are shown, one of which contains precipitate.

(c,d) *Sodium-rich tissue.*

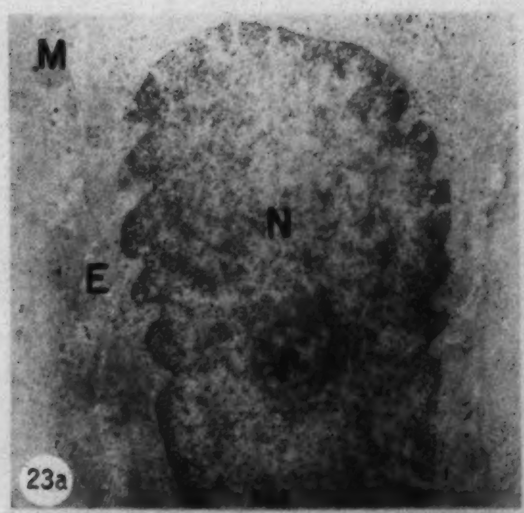
(c) Tissue fixed in 1% OsO_4. The pattern of precipitates is similar to that found in fresh tissue fixed in OsO_4.

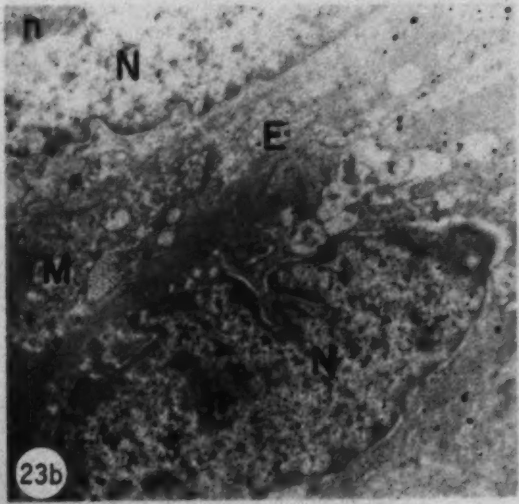
(d) Tissue fixed in 3% glutaraldehyde followed by 1% OsO_4. Precipitates are similar in size, location, and amount to those seen in fresh tissue fixed in glutaraldehyde.

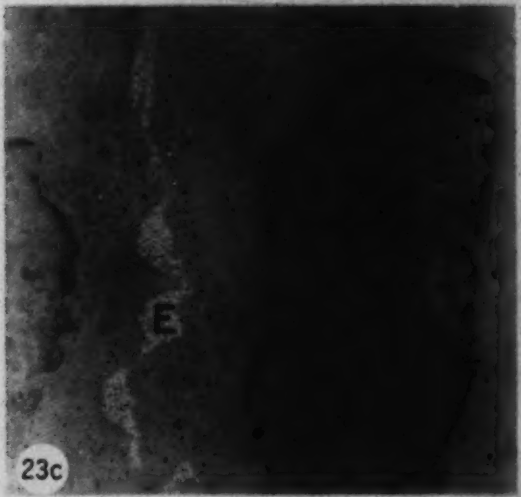
(e,f) *Sodium-poor tissue.*

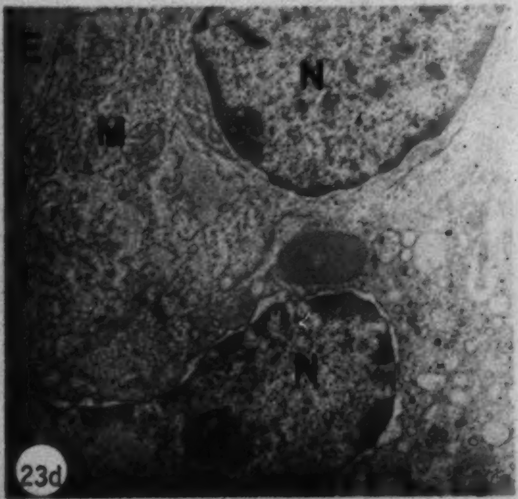
(e) Tissue fixed in 1% OsO_4.

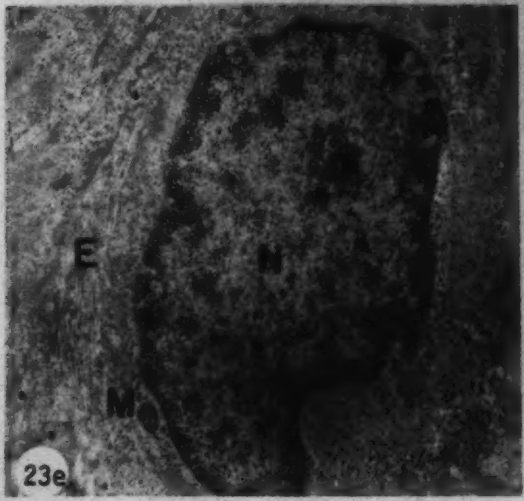
(f) Tissue fixed in 3% glutaraldehyde followed by OsO_4.

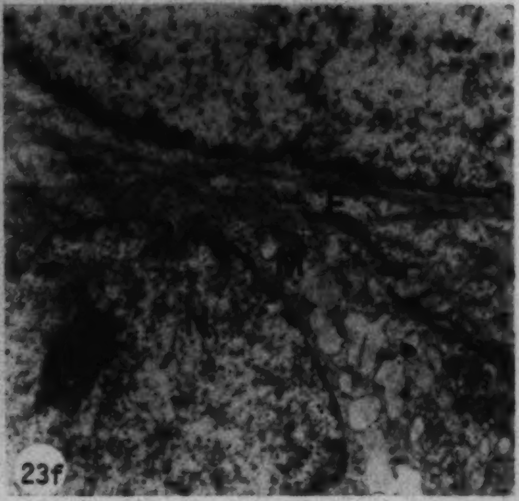

M
N
E
23a


N
E
M
N
23b


E
23c


E
N
M
N
23d


E
N
M
23e


E
23f

9. *Dry/Wet Weight Ratio*

Table 16 shows the final dry weight of tissues before or after fixation, divided by the initial fresh weight (dry/wet weight ratio). Although the ratios for controls were slightly higher than previously reported for the uterus (Moawad and Daniel, 1971), there was no consistent change in this ratio when tissues were fixed in OsO_4 or in glutaraldehyde alone or with pyroantimonate.

D. Discussion

1. *Reliability of Analytical Methods*

These studies provide strong evidence that pyroantimonate did not interfere with the methods used for determination of tissue cations. Spectrophotometric determination of known concentrations of cations were not affected by the presence of pyroantimonate provided their concentrations were low, as in the dilutions used for tissue analysis. The higher sodium and pyroantimonate concentrations, at which precipitates persisted after dilution, yielded reduced spectrophotometric readings; these concentrations are consistent with the known solubility of sodium pyroantimonate (30 mg/100ml or 680 μM; Hodgman [Editor], 1943). Values for the solubility of calcium pyroantimonate have not been found in any standard chemical reference. However, the calcium curves deviated at a point which would give approximately 10 μM calcium pyroantimonate. In the tissue used in

TABLE 16

Effect of fixative and pyroantimonate on the dry/wt. weight ratio

Exp.	Control	OsO_4	OsO_4 + Pyroantimonate	Glutaraldehyde	Glutaraldehyde + Pyroantimonate
1.	18.2 (17.0-19.4)	18.6 (18.0-19.6)	18.5 (17.5-19.2)		
2	20.7 (18.4-23.7)			20.8 (19.6-22.3)	20.1 (19.2-21.4)
3	20.6 (19.0-23.9)	19.9 (18.9-23.9)	21.5 (20.3-22.4)	19.5 (17.4-20.4)	19.6 (18.2-22.4)

Values shown are the dry weight of tissue divided by the initial fresh wet weight. The average is shown (expressed as %) with range of 4 values in parentheses.

this study (< 50 mg wet weight), the total amount of cations present yielded less than the above concentrations (160 μM NaCl and 6 μM $CaCl_2$) necessary to produce interference after dilution for analysis. Thus, if the tissue cations diffused out and into 1 ml of fixative containing pyroantimonate and the resultant solution was diluted to 25 ml, no interference with analysis would occur. Nor would interference be expected from tissue digested and diluted to 25 ml.

Analysis of both tissue and fixation and dehydration solutions showed that it was possible to account for all cations which were present in the tissue before fixation. This eliminates the possibility of formation of insoluble tissue precipitates which would not be detected in the analysis.

Data from ^{22}Na determinations of tissues fixed in OsO_4 or glutaraldehyde were similar to the results obtained for total tissue sodium based on spectrophotometric analysis.

2. *Inadequacies of the Pyroantimonate Technique*

The requirements for a valid histochemical method to localize tissue sodium by electron microscopy have been outlined by Bulger (1969) and include (1) the capacity to precipitate sodium quantitatively in the tissue before sodium can move; (2) a specificity for sodium; (3) the ability to permeate cell membranes and compartments; (4) the ability to form stationary, electron-dense precipitates with sodium. Pyroantimonate does not conform to these requirements when used to localize sodium in the uterus.

Quantitative considerations. Uterine tissues fixed in OsO_4 alone contained only about one-fourth of the original amount of sodium, which was approximately equal to the estimated intracellular content; those tissues fixed in the presence of pyroantimonate or in glutaraldehyde with or without pyroantimonate contained even less. The amount of sodium retained by fixed tissues was not increased nor decreased when the intracellular sodium content was altered before fixation. This indicates that even the first conditions required for quantitative determination of sodium were not fulfilled.

There was more ^{124}Sb-pyroantimonate in OsO_4-fixed tissues (ca. 40 times) than could be associated with sodium (assuming 2 moles Na^+ : 1 mole $Sb_2O_7^=$). At least 75% of the pyroantimonate might have been located within cells when fixed in OsO_4 (assuming no change in the extracellular volume; see calculation for intracellular sodium). On the other hand, pyroantimonate was present in much smaller amounts in glutaraldehyde-fixed tissues and may have been primarily extracellular in location. If this were so, then one might expect tissues to contain approximately 19 mmoles of ^{124}Sb-pyroantimonate per kilogram of tissue, based on the above assumptions. This value agrees with those obtained (ca. 20 mmoles; Figure 21).

The amount of tissue precipitate as seen in the electron microscope did not vary with the sodium content in the three types of tissues, confirming the results of sodium analysis. The amount and location of precipitates depended upon the method of fixation. Tissues fixed in OsO_4 contained precipitates throughout the tissue, both intracellularly and extracellularly, whereas fewer intracellular precipitates were observed after glutaraldehyde fixation. This is consistent with the analytical indications of the amount and the location of pyroantimonate after fixation. Furthermore, these findings are also consistent with observations made of kidney slices in which intracellular precipitates were observed only after OsO_4 fixation or fixation of swollen tissues with glutaraldehyde (Bulger, 1969). A similar difference in precipitate distribution with the two fixatives has been reported for cerebral cortex (Sumi, 1971; Sumi and Swanson, 1971) and for bone marrow cells (Clark and Ackerman, 1971a).

The inability of pyroantimonate to precipitate sodium quantitatively in the uterus did not agree with studies based on the semi-quantitative measurements of precipitates and the sodium content of embryonic chick heart (Klein *et al.*, 1970; Thureson-Klein and Klein, 1971). Furthermore, these results differed from qualitative observations which indicated that precipitates decreased after soaking tissue in sodium-free solutions (Edelhauser and Siegesmund, 1968; Bulger, 1969) or increased after incubation in sodium chloride solutions prior to fixation (Satir and Gilula, 1970). The reason for these differences probably was due to the qualitative nature of such studies.

The dry/wet weight ratios of fresh tissues were approximately 20%. There was no apparent increase in the ratios after fixation (Table 16). This suggests the loss of constituents from the tissues during fixation equal in weight to that gained from the fixative or pyroantimonate. The increase in weight seen by others after fixation in the presence of cations (Krames and Page, 1968) could perhaps be due to the gain in cations as well as fixative; or perhaps the presence of cations in the fixative increases the uptake of the fixative.

Permeability. For pyroantimonate to quantitatively precipitate tissue sodium, pyroantimonate must penetrate rapidly into the extracellular and intracellular spaces before sodium movement occurs. Based on its ionic size and charge, pyroantimonate would not be expected to enter tissue faster than sodium moves out into the fixative. Sodium is known to move rapidly into and out of cells from radioactive tracer studies. Pyroantimonate, in contrast to sodium, may not enter cells until after the membrane permeability is altered by the fixative. Penetration of the fixative, however, is known to proceed slowly from the surface to the inside of the tissue block, whereas sodium efflux from tissue occurs very rapidly. Therefore, sodium may diffuse out of the tissue before pyroantimonate diffuses in with the fixative.

Solubility. Even if pyroantimonate ions were able to diffuse into tissue and cells instantaneously before sodium movement occurred, it should still form insoluble precipitates with all sodium to quantitatively localize cell sodium. Sodium pyroantimonate, however, has a finite solubility product so that not all sodium would be precipitated. Using the value of 400 mmoles Na^+/kg dry weight (ca. 80 mmoles Na^+/kg wet weight) for fresh uterine tissues (Figure 17) and the solubility coefficient of sodium pyroantimonate (30 mg/100 ml, K_{sp} 2 x 10^{-10}; Hodgman [Editor], 1943), one would calculate that only 70% of the total sodium in a 50 mg piece of tissue would form insoluble precipitate when placed in 1 ml of solution.

The possibility of 90-95% of tissue sodium precipitation with pyroantimonate, which is based on greater solubility of sodium pyroantimonate ($K_{sp} = 2 \times 10^{-6}$) (Klein *et al.*, 1972) than reported by standard solubility references, is probably incorrect. Furthermore, increasing the volume of the fixative, as proposed by Klein and Thureson-Klein (1972) to prevent pyroantimonate ion from becoming limiting, would only increase the amount of sodium which could be in solution. In order for pyroantimonate ion (40 moles/ml) to become limiting, a piece of tissue weighing more than one gram (80 moles Na^+) must be used in 1 ml of fixative.

Specificity. Calcium and magnesium are less mobile in tissue than sodium, and also form precipitates with pyroantimonate in test tubes (Bulger, 1969). Fixed uterine tissues contained some calcium and nearly their normal complement of magnesium after fixation and dehydration. That they contribute to precipitates seen in other tissues examined with the electron microscope is supported by electron probe analysis (Tandler and Kierszenbaum, 1971), but they cannot account for all ^{124}Sb-pyroantimonate retained by uterine tissues.

3. *Nature of Precipitates Seen in the EM*

Potassium. It seems reasonable that all the pyroantimonate which remained in fixed tissue was precipitated, since little washed out during dehydration. If so, approximately one-half of the precipitates in OsO_4-fixed and all precipitates in glutaraldehyde-fixed tissues could be potassium, based on the content of ^{124}Sb-pyroantimonate and potassium retained. Test tube experiments showed, as confirmed by others (Tice, personal communication; Shiina *et al.*, 1970), that potassium pyroantimonate was insoluble in dehydrating solutions such as alcohol, acetone and propylene oxide. Consequently, potassium pyroantimonate precipitates may be formed in the tissue during dehydration. In addition, potassium precipitates may be formed in the tissue due to changes in pH, since potassium pyroantimonate solutions precipitate spontaneously upon lowering the pH to 6.8 (Torack and LaValle, 1970; Shiina *et al.*, 1970). The pH at which potassium pyroantimonate precipitates could be close to the intracellular pH or that pH of some organelles.

Additional evidence that deposits represent potassium comes from the observation that potassium has the same distribution as antimony in tissue precipitates studied by electron probe analysis (Tandler and Kierszenbaum, 1971).

Shiina *et al.* (1970) have suggested that potassium might be removed after fixation by washing. However, little loss of potassium was observed in uterine tissues washed in distilled water after fixation or during dehydration. Tandler *et al.* (1970) heated fixed tissues in half-saturated potassium pyroantimonate solution to remove only the precipitated potassium and retain other precipitates by a common ion effect. They noted no appreciable decrease in tissue precipitates and concluded that the precipitates were not potassium. The value of this procedure is doubtful since this method might have failed to remove all the potassium pyroantimonate which subsequently precipitated during dehydration.

The potassium concentration in the fixative is increased when potassium phosphate buffer is used to control the pH of fixative-pyroantimonate solutions. When potassium was added to fixatives containing pyroantimonate either as phosphate buffer of chloride, Shiina and coworkers (1970) observed a decrease in tissue and test tube precipitates. They proposed that the formation of sodium pyroantimonate was inhibited by coexisting potassium ions in the fixative and they recommended unbuffered fixative for sodium detection. Torack and LaValle (1970) attributed the decrease in tissue precipitates to an interaction of phosphate anion with the pyroantimonate anion thereby decreasing the sites available for sodium to react. They did not specify how the two anions interact. They recommended the use of phosphate buffered pyroantimonate fixative and artificial elevation of sodium ions, since they found potassium was precipitated in test tubes at physiological levels in unbuffered solutions. Klein *et al.* (1972) do not think that physiological levels of potassium contribute to precipitate formation, based on test tube studies. Whether or not potassium pyroantimonate is precipitated by physiological levels of potassium does not eliminate the possibility of formation of tissue precipitates during dehydration. Unbuffered fixatives were used in this study because inorganic buffer salts (particularly potassium phosphate) might allow ion exchange between tissue and fixative and organic buffers might be oxidized by, and decrease the action of, the fixative.

Sodium. Some of the residual sodium which remained in tissue after fixation and dehydration could be associated with pyroantimonate. This would explain results obtained with autoradiography (Tisher *et al.*, 1969), electron probe analysis (Lane and Martin, 1968; Tandler *et al.*, 1970; Kierszenbaum *et al.*, 1971), and selected area diffraction (Hartman, 1966). However, the presence of other precipitates which might be indistinguishable from sodium precipitates in size and density reduces the usefulness of this technique even for the localization of residual sodium.

Tandler *et al.*(1970) and Kierszenbaum *et al.* (1971) reported massive precipitation of cations and good tissue preservation following immersion and perfusion of tissue in 2% potassium pyroantimonate solution at pH 9.2. They propose that pyroantimonate behaves as a fixative. Obviously, potassium pyroantimonate is not useful alone to precipitate sodium in the uterus, since little sodium remained after incubation of tissue in pyroantimonate sodium (Table 15). Furthermore, uterine tissues did not have the physical appearance of fixed tissue after incubation in pyroantimonate solutions as they were indistinguishable from fresh tissue. This may indicate that the tissues were not fixed by pyroantimonate. Moreover, the above workers always hardened their tissues in formaldehyde after pyroantimonate treatment. I suggest that pyroantimonate, by itself, does not act as a fixative on myometrium.

Calcium and magnesium. Some of the precipitates could be calcium which was not completely lost or magnesium which was retained by uterine tissues after fixation and dehydration. Legato and Langer (1969) have proposed that the pyroantimonate method is valid for the intracellular localization of these two divalent cations. They support their contention with the observation that intracellular precipitates in dog papillary muscle were removed when tissues were incubated in EGTA and EDTA solution prior to fixation.

In embryonic heart, Klein *et al.* (1970) observed decreased nuclear, cytoplasmic, and mitochondrial precipitates after fixation of tissue incubated in high concentrations of EGTA designed to remove both calcium and magnesium. They concluded from semi-quantitative measurements that 40% of tissue precipitates were sodium and the remainder, calcium and magnesium. However, if calcium, magnesium and sodium were precipitated quantitatively, one would expect more than 75% of the precipitates to be sodium. More recently they suggested the use of chelating agents to remove divalent cations when only sodium is to be demonstrated (Klein *et al.*, 1972). It seems unlikely that a chelating agent would remove divalent cations from tissue while the use of a precipitating agent would not remove sodium.

Further evidence that calcium and magnesium contribute to precipitates seen in tissues is provided by electron probe analysis studies (Tandler and Kierszenbaum, 1971). These studies indicate that tissue precipitates contain calcium and magnesium as well as sodium and potassium. Furthermore, empty spaces in sections have been noted after floating them on oxalate solutions designed to remove calcium (Kierszenbaum *et al.*, 1971).

In uterine tissues fixed in OsO_4, one-fourth of the pyroantimonate precipitate may represent calcium, magnesium and other residual ions in addition to that which may react with potassium, based on their contents in fixed tissue.

Organic precipitates. There was more ^{124}Sb-pyroantimonate retained by OsO_4-treated tissues (ca. 20 mmoles ^{124}Sb-pyroantimonate/kg tissue wet weight or one-fourth of the total amount) than could be precipitated by all the remaining tissue cations. Therefore, the remaining one-fourth or more of the pyroantimonate in OsO_4-fixed tissues was likely precipitated by tissue constituents other than cations. These results support evidence that pyroantimonate may react with other substances such as biogenic amines, glycogen, and amino groups of nucleic acids (Bulger, 1969; Clark and Ackerman, 1971b).

4. *Effect of Fixatives on Membrane Permeability*

The sodium content of tissues fixed in OsO_4 was approximately equal to the intracellular content before fixation. The loss of intracellular sodium from tissues fixed in OsO_4 containing potassium pyroantimonate may depend upon a sodium-potassium exchange across the membrane or at binding sites. Tissues contained less sodium after exposure to solutions or fixatives containing high potassium concentrations (Table 15). Pyroantimonate, on the other hand, does enter cells fixed in OsO_4, as indicated by precipitate location and ^{124}Sb-pyroantimonate content. This might be a consequence of OsO_4 converting the cell membrane from one with fixed anionic charges and capable of exchanging cations, to one containing some fixed cationic charges and capable of exchanging anions. The sodium content was lower in glutaraldehyde-fixed tissues. Glutaraldehyde fixation, in contrast to OsO_4 fixation, might affect the membrane in a manner that allows the loss of intracellular cations (sodium and potassium were both lower after glutaraldehyde fixation than after OsO_4 fixation) but limits the entry of pyroantimonate. Whatever the explanation, the two fixatives clearly have a different effect on membrane permeability. Further work is necessary to clarify these findings.

5. *Utilization of Pyroantimonate for Localizing Tissue Sodium*

In light of these results, the pyroantimonate method cannot be used to localize sodium quantitatively in the uterus which would be necessary to demonstrate sodium pumping sites. Furthermore, unless other tissues can be shown to behave differently from the uterus, the pyroantimonate technique should be abandoned.

CHAPTER 4

LIGHT AND DARK SMOOTH MUSCLE CELLS

CHAPTER 4

LIGHT AND DARK UTERINE SMOOTH MUSCLE CELLS

A. Objectives

Two distinct populations of cells were observed in preliminary examinations of rat uterine smooth muscle fixed by immersion in glutaraldehyde. Among other distinguishing characteristics, the cells of one population were less electron dense and contained no membrane vesicles as compared with the other population. Since smooth muscle cells without membrane vesicles may be unable to maintain their volume (Chapter 2), the two types of cells were studied.

The objective of this study was to define the two types of cells more precisely in ultrastructural terms and to investigate their origin and significance by answering the following questions: (1) What are the morphological differences between the two cell types, and what do these differences indicate? (2) What factors affect the relative numbers of light and dark cells and what can we infer about the nature of these two cell types from study of variables which influence their relative numbers?

B. Materials and Methods

1. *Animals*

Estrogen-dominated rats. Immature female (Wistar strain) rats weighing less than 100 grams were injected subcutaneously with 50 micrograms of diethylstilbesterol daily for 6 days.

Ovariectomized rats. Immature rats weighing less than 100 grams were anesthetized with pentobarbital and ovariectomized. The rats were allowed to recover from the trauma for one week and then treated as described below.

Mature-pregnant rats. Pregnant rats (near term, 30 days gestation weighing approximately 280 grams were used in this investigation.

Tissue preparation. Except when tissues were to be fixed *in situ* (see below), the tissues were treated as described in Chapter 2.

Contraction or relaxation of tissues was studied after attaching one gram weights to the cervical end of uterine horns. To relax these tissues, they were incubated in Krebs-Ringer solution with adrenaline (1×10^{-7} grams/ml) for several minutes. Oxytocin (0.001 unit/ml) was used to produce contraction. When maximum relaxation or contraction was obtained, the uteri were transferred to fixative. The paired horns were also weighted but not treated. Measurements of initial, intermediate, final and fixed lengths of tissues were made with a compass and millimeter scale.

2. *Solutions*

The Krebs-Ringer solution used in this study contained the same constituents as that indicated in Chapter 2, page 34. Metabolic inhibitors were added directly to the Krebs-Ringer solution in concentrations of 1 mM.

3. *ATP Estimation*

ATP determinations of tissues were accomplished by the firefly method as described in Chapter 2.

4. *Electron Microscopy*

Tissue preparation. Some tissues were fixed by immersion in 5 ml of 5% glutaraldehyde at 25°C (pH 7.4; 950 mOsm) with phosphate buffer (Millonig, 1962). Other tissues of anesthetized animals were fixed by slow perfusion of the uterus with 5%, phosphate buffered glutaraldehyde through cannulae inserted in the direction of blood flow in the abdominal aorta. *In situ* fixation was considered complete when the uteri changed color from its normal pink to deep yellow. All glutaraldehyde-fixed tissues were stored in this solution in the cold (24 to 48 hours) until ready for post-fixation. Post-fixation was carried out for 1 hour in 1% osmium tetroxide with phosphate buffer.

Additional tissues were fixed for 1 hour by immersion in 1% potassium permanganate (pH 7.0; 280 mOsm) or buffered 1% osmium tetroxide followed immediately by dehydration. Potassium permanganate fixative was prepared by diluting a 2% permanganate solution to 1% with 0.9% NaCl solution. One percent osmium tetroxide fixative was made by dissolving 0.1 gram of OsO_4 in 10 ml of Millonig's buffer.

After fixation, tissues were prepared as previously described in Chapter 2.

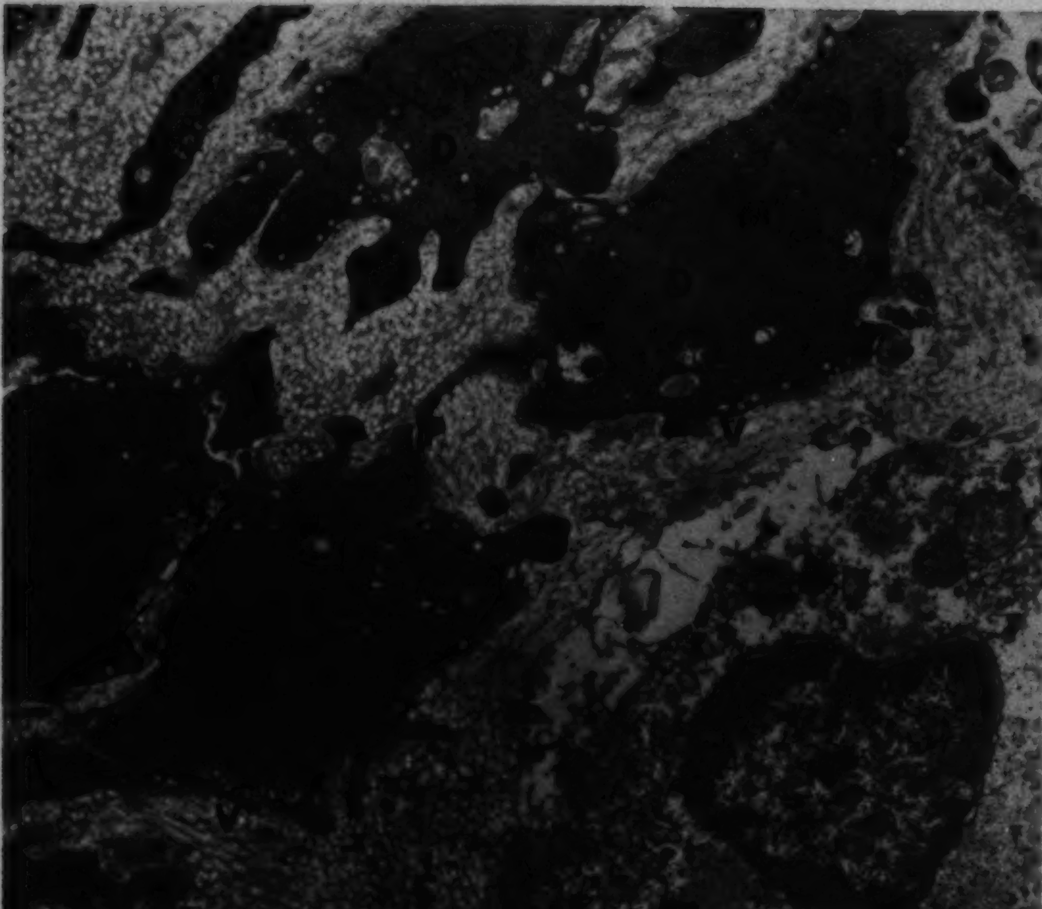
Quantitative studies. The relative number of light and dark cells was determined by either one of two methods: (1) Light and dark cells were counted on the EM screen by scanning sections overlying four different grid squares (the center squares from each quadrant of 300 mesh grids) at 8,000 magnification. (2) Cell counts were made from 8" x 10" photographs (x 24,000 magnification) taken according to the procedure described in Chapter 2. Light smooth muscle cells were distinguished from dark cells on the basis of several characteristics (Table 17).

C. Results

1. *Appearance, Location and Number of Light and Dark Cells*

Figure 24 shows the general appearance of smooth muscle cells of the outer longitudinal muscle layer of an estrogen-dominated rat myometrium fixed by immersion in glutaraldehyde. Two types of cells can be recognized in reference to electron density. These cells have been termed light and dark cells after their respective electron densi-

Figure 24. Smooth muscle cells from the longitudinal layer of an estrogen-dominated rat uterus incubated in Krebs-Ringer solution for 60 minutes and fixed by immersion in 5% glutaraldehyde followed by post-fixation in 1% OsO_4. Light (L) and dark (D) cells can be recognized primarily by differences in cytoplasmic density. Note the cytoplasmic projections (P), the small mitochondria (M), the darker nucleoplasm in the nucleus (N) and membrane vesicles (V) in the dark cells (D) in contrast to these structures in light cells (L). The endoplasmic reticulum (ER) is swollen in the light cells. Collagen (C) occupies the extracellular space. x 12,500.


24

ties (Gansler, 1961), or round or star-shaped after their surface appearance (Aita *et al.*,1968a) in uterine and other smooth muscle tissues.

In addition to different densities, light and dark smooth muscle cells of the uterus have numerous other structural differences summarized in Table 17. In general, light smooth muscle cells have a swollen appearance as indicated by swollen endoplasmic reticulum, swollen nuclear envelope and rounded mitochondria with orthodox configuration (Hackenbrock, 1966) in contrast to the structure of dark cells (Figure 24, Table 17).

Table 18 shows the number of light and dark cells found in the rat uterus after various treatments. Light cells accounted for approximately 4% of the total population of muscle cells in the longitudinal layer of both tension-loaded and unloaded estrogen-dominated rat myometrium (Groups 2,4,6). They were found randomly distributed among dark cells when whole uterine tissues were examined. Light cells were more frequent along the stripped circular muscle layer after removal of the endometrium (Group 7). This treatment did not, however, affect their number in the outer longitudinal muscle layer (Group 6).

2. *Effect of Relaxation or Contraction*

To determine if relaxation or contraction alters the number of light and dark cells or their structure, loaded (1 gram) uterine horns were incubated in adrenaline (1 x 10^{-7} g/ml) or oxytocin (0.001 unit/ml) prior to fixation.

TABLE 17

Comparison of the structure of light and dark uterine smooth muscle cells fixed by immersion in glutaraldehyde.

Structure	Dark cells	Light cells
Nucleus		
Chromatin	Condensed in periphery	Condensed in periphery
Nucleolus	Present	Present
Nuclear pores	Nuclear pores seen only at high magnification	Numerous pores
Nuclear envelope	Membranes parallel, close	Swollen
Mitochondria		
Profiles	Various shapes typically elliptical	Round
Conformations	Condensed	Orthodox
Golgi apparatus	Present	Present
Endoplasmic reticulum	Compact membranous structures with associated ribosomes	Swollen with many attached ribosomes
Muscle filaments	Many thin filaments oriented in long axis of cell	Few filaments
Cell surface		
Cytoplasmic projections	Numerous narrow and pointed	Blunt projections on some, none on others
Plasma membrane	Continuous, triple layered	Not well preserved, continuity lacking
Plasma vesicles	Numerous vesicles located along the plasma membrane	Very few or none

TABLE 18

Effect of various treatments on the number of light and dark uterine smooth muscle cells

Group	Hormonal State of Uteri	Fixative & Method		Total Cells Counted*	Dark Cells %	Light Cells %
	Estrogen dominated	Glutaraldehyde immersed	Whole uteri-tension loaded			
1			Relaxed with adrenalin	1280	98	2
2			Untreated control	1068	96	4
3			Contracted with oxytocin	878	92	8
4			Untreated control	1103	96	4
5			Circular muscle layer	1340†	89	11
			Endometrium removed			
6			Longitudinal muscle layer	1437	95	5
7			Circular muscle layer	1230	63	37
8			Cells on the stripped surface	760	-	100
9	Overiectomized rat uteri	------------------------------	Whole Uteri	830†	93	7
10	Pregnant rat uteri			520†	98	2
11	Estrogen dominated uteri	OsO_4 immersed	Endometrium Removed	890†	Only one type of cell could be found with OsO_4 or $KMnO_4$	
12		$KMnO_4$ immersed		640†		
13		Glutaraldehyde perfused *in situ* -------	Whole Uteri	1286	100	-

* Total cells counted from 4 separate tissues.

Groups 1,2,3,4,6,13 are values obtained from the longitudinal muscle layer oriented in cross-section.

† Counts were made from muscle cells in longitudinal orientation.

Tissues shortened 33% (30 - 35%, n = 4) of their initial length measured under 1 gram of tension when treated with oxytocin. Relaxation with adrenaline produced an additional 7% (5 - 9%, n = 4) increase in initial-loaded length. No measurable changes in contracted or relaxed lengths occurred upon fixation of tissues.

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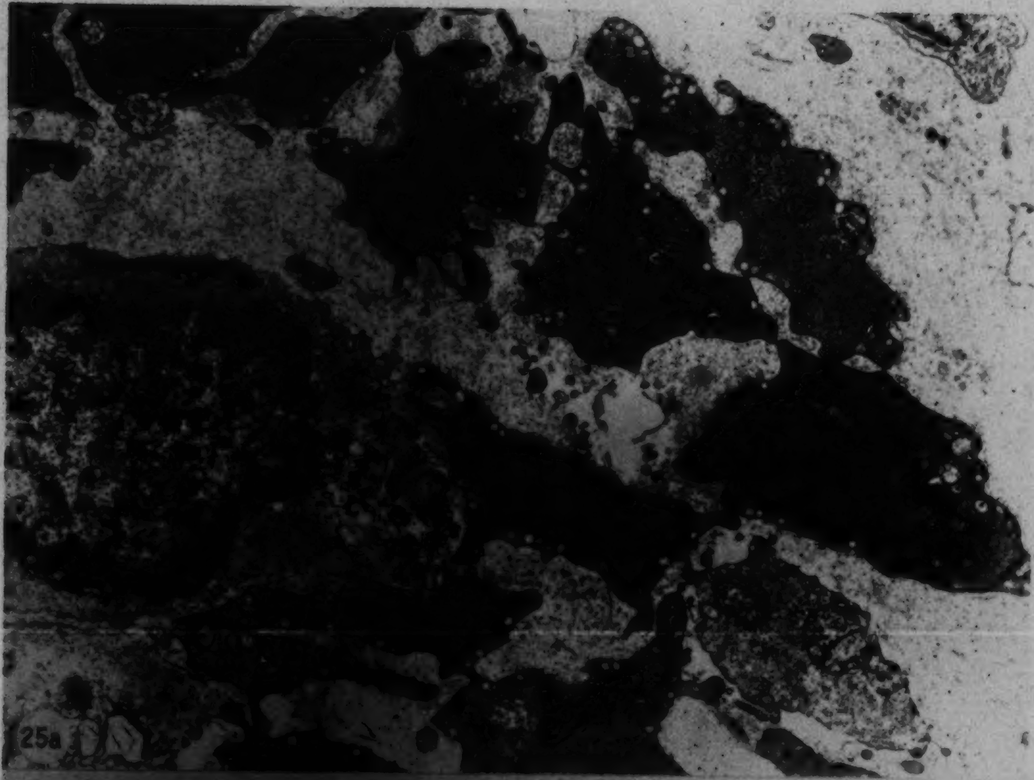
Relaxed dark smooth muscle cells (Figure 25a) had fewer cytoplasmic projections and the cross-sectional diameter was less than contracted dark cells (Figure 25b). There were only slight differences in the relative number of light and dark cells when contracted tissue was compared with relaxed tissue or when treated tissues were compared with their untreated controls (Table 18, Groups 1-4). However, there were more light cells in the contracted tissues than in the relaxed tissues although dark cells were predominant after both treatments.

3. *Effect of Immersion in Other Fixatives*

The light and dark cells described above were found after immersion fixation in glutaraldehyde. Tissues were examined after immersion in potassium permanganate or osmium tetroxide to see if light and dark cells could be demonstrated with these fixatives.

Smooth muscle fixed by immersion in potassium permanganate is shown in Figure 26a. Only one type of cell could be distinguished after permanganate fixation (Table 18, Group 12). The characteristic differences which distinguished light and dark cells found after glutaraldehyde fixation (Table 17) were absent. In all cells, the chromatin appeared uniformly distributed throughout the nucleus as did the

Figure 25. Cross-sections of longitudinal muscle cells from estrogen-dominated uteri: (a) Relaxed in adrenaline prior to fixation. (b) Contracted with oxytocin prior to fixation. x 8,400. Note the presence of light cells in both conditions and the increased cross-sectional diameter of the contracted cells.


25a

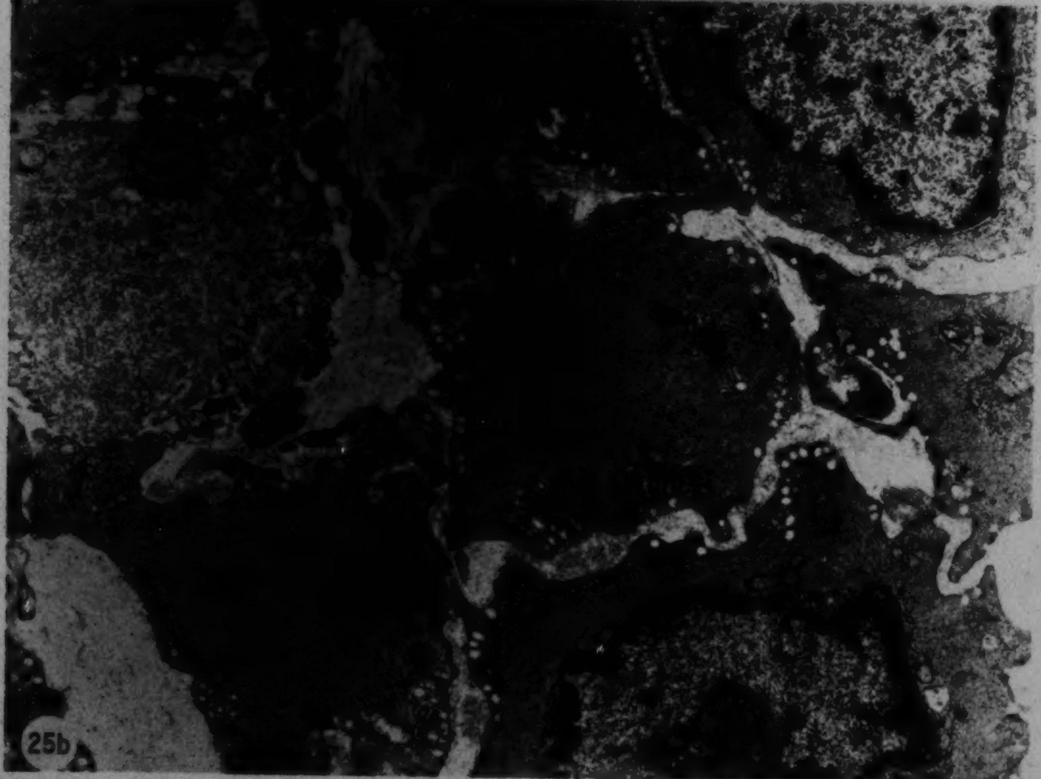
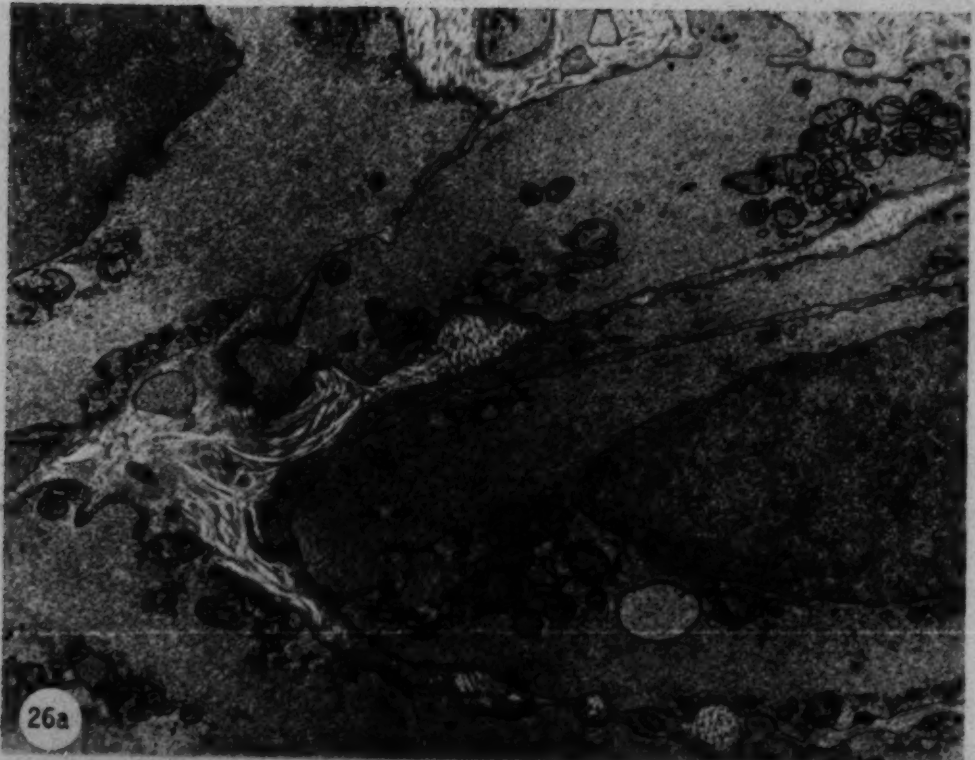

25b

Figure 26. Structure of smooth muscle after potassium permanganate and osmium tetroxide fixation. (a) Fixation by immersion in 1% potassium permanganate. Note the good preservation of membrane and lack of other cytoplasmic (filaments) or nuclear (chromatin) structures as seen after glutaraldehyde fixation. The membrane vesicles were smaller and often absent. x 8,000. (b) Smooth muscle after immersion in 1% osmium tetroxide. Membrane preservation is similar to that of permanganate fixed tissues (as above) but cytoplasmic and nuclear structures are more apparent. x 8,400. Light and dark cells as defined for glutaraldehyde-fixed tissues could not be found in either permanganate or osmium-fixed tissues.



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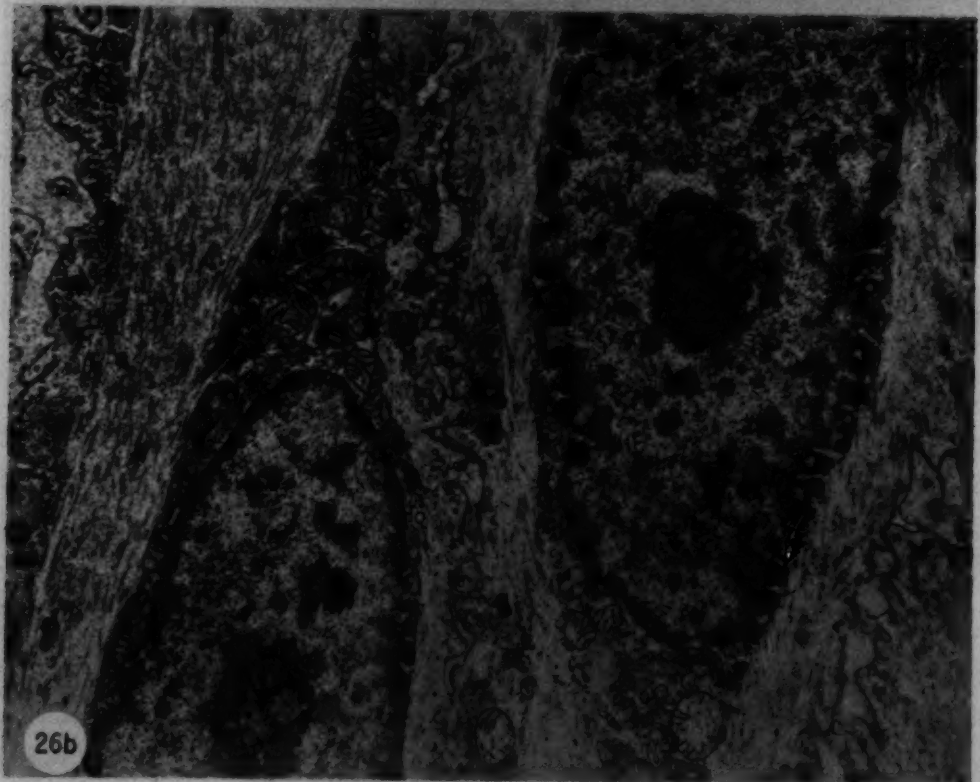


26b

ground substance in the cytoplasm. All membranes were well demonstrated; however, membrane vesicles were noticeably smaller and often absent as compared to cells fixed in glutaraldehyde. The mitochondria were all rounded with orthodox conformations and membrane projections were less prominent. Therefore, these cells had some characteristics in common with light cells.

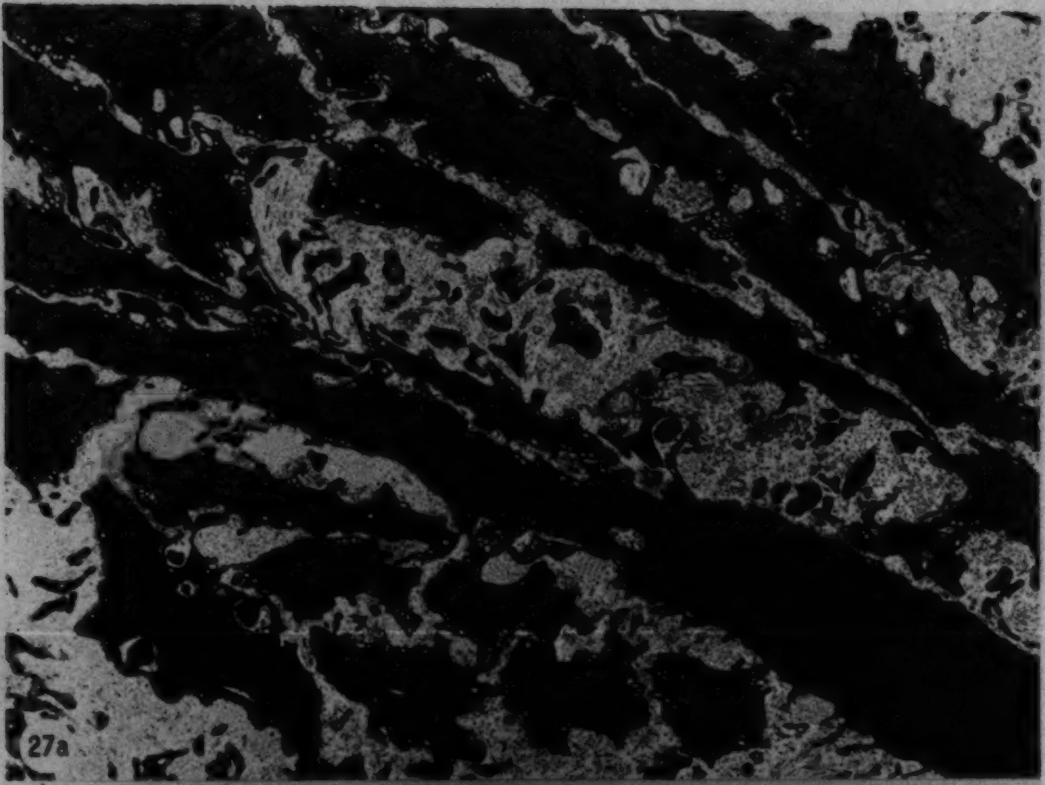
Like tissues fixed in potassium permanganate, tissues fixed by immersion in osmium tetroxide were found to contain only one type of smooth muscle cell (Figure 26b, Table 18). The chromatin material was clumped as in light cells found after glutaraldehyde fixation (Figure 24). The mitochondrial and plasma membrane of OsO_4-fixed cells were similar to that seen after permanganate fixation.

4. *Structure of Immature and Mature Uteri*

The uterus is known to respond to estrogen stimulation by increasing the number and size of its cells. The question posed here was whether light cells represent newly differentiated or immature cells. If so, then one would expect to find more light cells in immature and immature estrogen stimulated uteri in contrast to the number in mature tissues.

Uterine smooth muscle cells were small in myometria from immature ovariectomized rats (Figure 27a). Much larger cells, which were similar to cells found in estrogen-stimulated uteri (Figure 24). were found in uteri from pregnant rats (Figure 27b). There were no or only slight differences in the relative number of light and dark

__Figure 27__. Longitudinal sections of dark cells from ovariectomized and pregnant rats. Glutaraldehyde immersion. (a) Muscle cells from immature ovariectomized rat (without estrogen) x 8,400. (b) Myometrium from near term pregnant rat. x 8,400. Note the difference in size between the cells from the immature and mature uterus.



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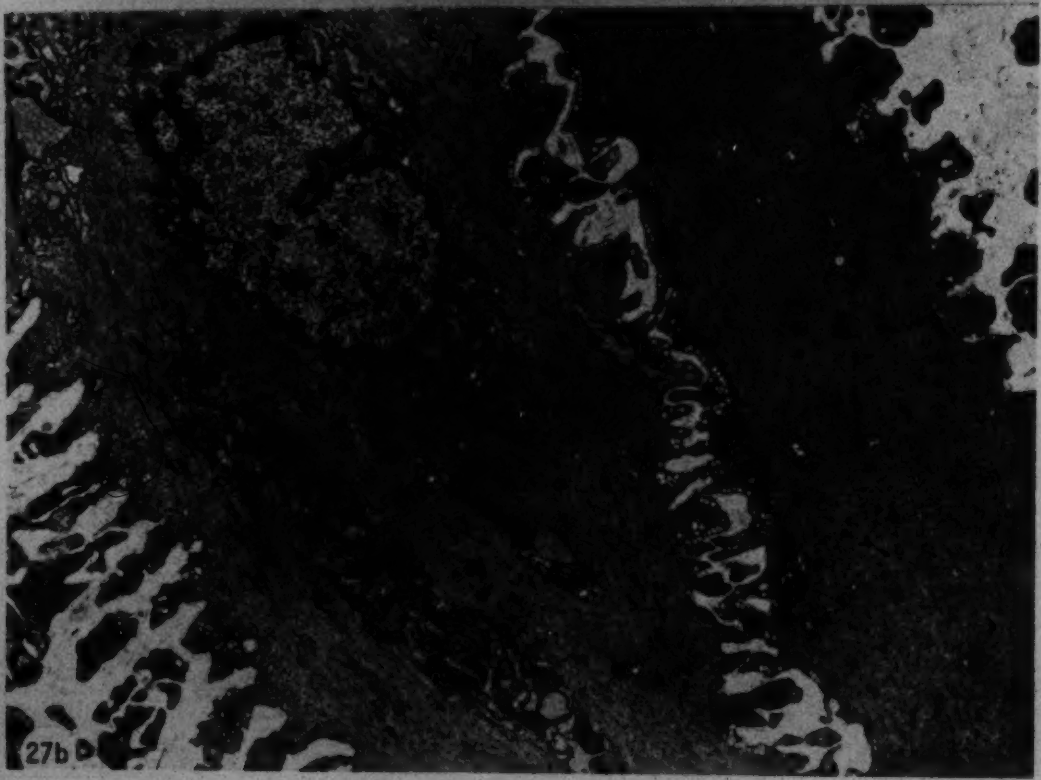


27b

cells in these uteri (Table 18, Groups 9 and 10).

5. *In situ Glutaraldehyde Fixation*

Uteri fixed by intra-arterial glutaraldehyde perfusion were examined to determine if light cells were a manifestation of immersion fixation.

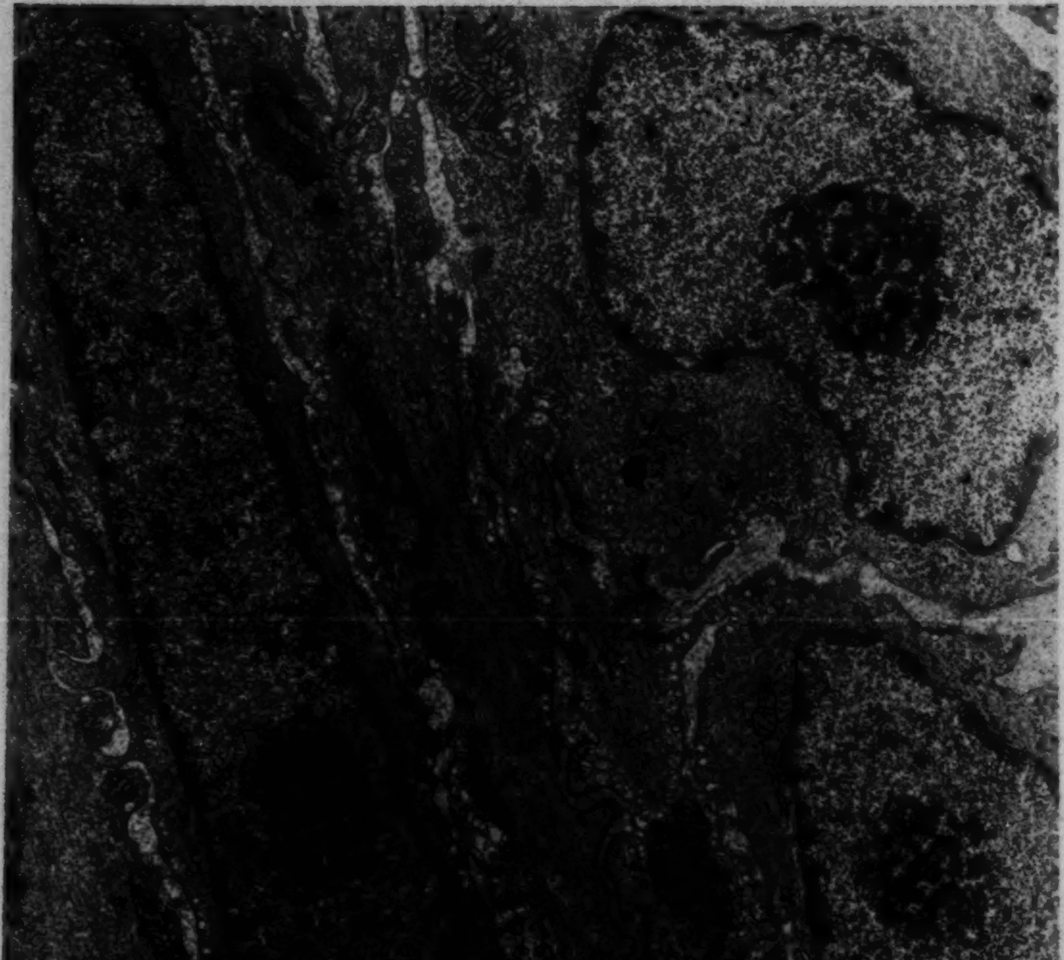
In situ fixation of uteri from estrogen-dominated rats resulted in cells which could only be described as dark cells (Figure 28). All cells had characteristics, such as membrane vesicles, elliptically shaped mitochondria and cytoplasmic projections, similar to dark cells found in tissue fixed by immersion (Table 18, Group 13). This method of fixation resulted in better tissue preservation than immersion fixation, as judged by less chromatic clumping and better detailed mitochondrial and membrane structure.

6. *Effect of Metabolic Inhibition*

Orthodox mitochondrial configuration, swollen endoplasmic reticulum and lack of membrane vesicles in light cells suggested that there may be underlying metabolic differences between light and dark cells. To study metabolic differences, tissues were examined after incubation with metabolic inhibitors.

Tables 19 and 20 show the number of light cells, content of ATP, and H_2O content in tissues at various times after metabolic inhibition with iodoacetate + dinitrophenol and iodoacetate alone. After the recovery period following uterine removal, 96% of the cells were

Figure 28. Estrogen-dominated rat myometrium fixed by intra-arterial perfusion with glutaraldehyde. The structure of cells is similar to dark cells found after immersion fixation. x 12,000.


28

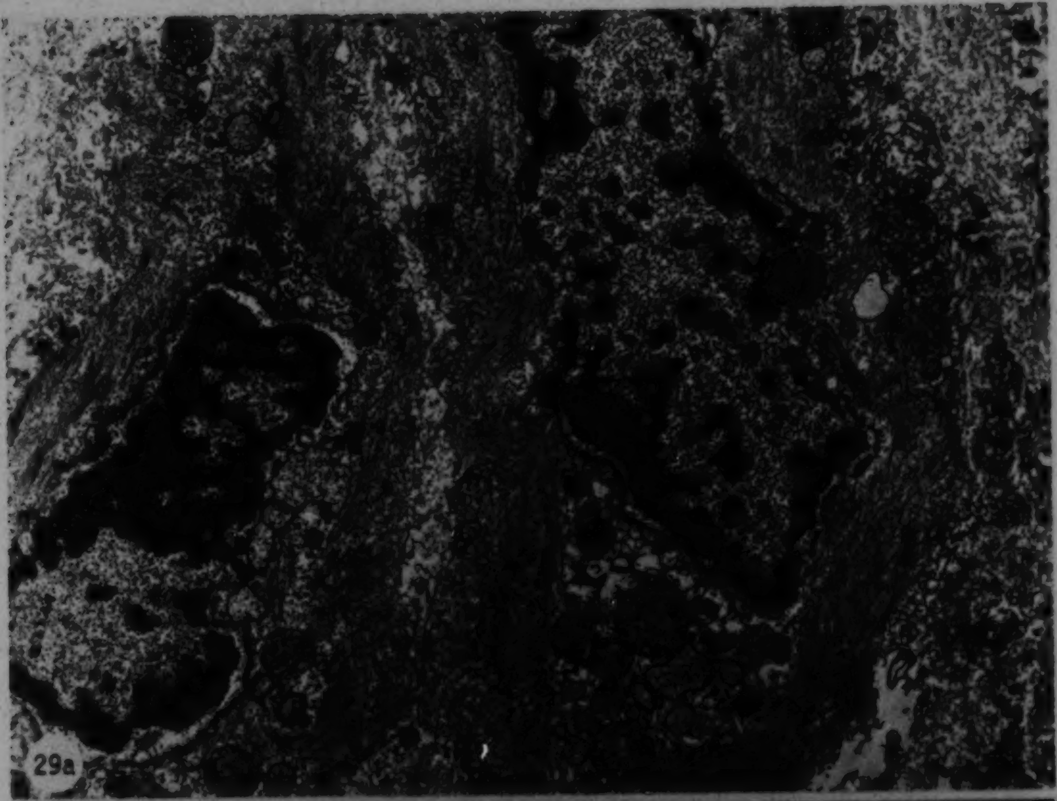
found to be dark cells. Following the decrease in ATP content of inhibited tissues, the population of light cells increased at the expense of dark cells. The time differential between ATP ($< 10\%$ of the control) and dark cell reduction was approximately 40 minutes with IAA + DNP and 80 minutes with IAA alone. There were no changes in the number of dark cells in the control paired uterine horns incubated in Krebs-Ringer without inhibitors over the same time interval. The water content of inhibited tissues increased after the shift from dark to light cells. The structure of smooth muscle cells after metabolic injury is shown in Figure 29a.

7. *Effect of Mechanical Injury*

To determine if injury by mechanical means would result in light cells, sections were made of the circular layer of the myometrium adjacent to where the endometrium was removed.

There were slightly more light cells in the circular muscle layer than in the longitudinal layer in whole myometrium (Table 18, Groups 2,4, and 5), the stripping procedure resulted in increased number of light cells in the adjacent circular muscle (Table 18, Group 8). The structure of these mechanically injured cells (Figure 29b) was similar to the structure of metabolically inhibited cells (Figure 29a).

Figure 29. Effect of chemical and mechanical injury on the structure of rat myometrium. Note the lack of membrane vesicles, the mitochondrial appearance and swollen endoplasmic reticulum: (a) Muscle cells after ATP depletion with IAA + DNP (80 minutes treatment). x 12,000. (b) Circular muscle cells on the edge of the tissue near the area of endometrial removal. x 12,000.



29a

29b

TABLE 19

Effect of IAA + DNP (1 m*M* each) on ATP content, H_2O content and the number of light and dark cells in the longitudinal muscle from rat myometrium

Treatment		Total Cells Counted[a]	Light Cells (%)	ATP[b] (% Control)	H_2O (g/g solids)
2.5 Min	Control	103	6	Not measured	4.15 (0.18)
	Inhibited	120	4		3.88 (0.18)
5 Min	Control	113	3	10	3.95 (0.21)
	Inhibited	119	15		4.40 (0.28)
10. Min	Control	104	7	6	4.11 (0.25)
	Inhibited	131	5		4.34 (0.35)
20 Min	Control	121	1	3	4.14 (0.18)
	Inhibited	105	42		4.48 (0.20)
40 Min	Control	-	-	0.5	4.17 (0.11)
	Inhibited	100	46		4.86 (0.12)*
60 Min	Control	128	5	Nil	4.12 (0.18)
	Inhibited	101	90		4.65 (0.23)*
80 Min	Control	98	3	Nil	4.16 (0.11)
	Inhibited	103	96		4.81 (0.04)*

[a] Data from 16 photographs at each time from 4 experiments.

[b] ATP contents from one paired control experiment.

[c] Mean values with S.E. in brackets of H_2O contents of tissues from 4 experiments.

* $P < 0.5$ using paired t-test.

TABLE 20

Effect of IAA on the number of light and dark cells

Treatment		Total Cells Counted	Light Cells (%)	ATP (% control)	(g/g H_2O solids)
20 Min	Control	162	2		3.95 (0.17)
	Inhibited	136	6	83	4.01 (0.10)
40 Min	Control	-	-		4.01 (0.10)
	Inhibited	204	16	10.5	4.35 (0.15)
60 Min	Control	128	2		4.30 (0.15)
	Inhibited	149	7	2	4.30 (0.15)
80 Min	Control	102	1		4.15 (0.26)
	Inhibited	175	18	0.3	4.23 (0.16)
100 Min	Control	160	0		4.23 (0.25)
	Inhibited	128	19	Nil	4.53 (0.29)*
120 Min	Control	131	2		3.99 (0.08)
	Inhibited	146	56	Nil	4.75 (0.11)*
150 Min	Control	154	3		3.90 (0.19)
	Inhibited	164	95	Nil	4.75 (0.11)*

Data indicated as in Table 19

D. Discussion

The findings can be briefly summarized as follows: Both light and dark smooth muscle cells were found in the same myometrial preparation following immersion fixation in glutaraldehyde and post-fixation in osmium tetroxide. Light cells were distinguished from dark cells by differences in nuclear, mitochondrial, endoplasmic reticular and surface structure. Tissues fixed *in vitro* after dissection and recovery consisted of approximately 96% dark cells and 4% light cells. Contraction or relaxation of tissues before fixation previously thought responsible for the two types of cells (see below), did not change the proportion of light and dark cells. Only dark cells were found after *in situ* fixation of tissues. After chemical or mechanical injury, smooth muscle was composed of light cells. This indicated that light cells may be cells which were injured prior to fixation or inadequately fixed during immersion fixation.

1. *Incompatability of Data With Previous Theories*

Contraction-relaxation. For over 180 years, investigators have described light and dark smooth muscle cells as viewed through the light microscope (see McGill, 1909; and Conti *et al.*, 1972 for review of early work). With the introduction of the electron microscope and improved methods of fixation and preparation, the presence of light and dark cells in smooth muscle (Gansler, 1961; Conti *et al.*, 1972), brain and liver (Ganote and Moses, 1968; Dohrman, 1971) were confirmed.

Light cells viewed under the light microscope were the same cells as identified under the electron microscope as both observations are dependent upon cell density.

Contraction and relaxation produced by mechanical or chemical means was thought responsible for increasing or decreasing the density of smooth muscle cells (McGill, 1909; Gansler, 1961; Conti *et al.*, 1972).

Perhaps cells are more or less dense when contracted or relaxed prior to fixation, although no one has yet provided quantitative measurements of this. If quantitative comparisons are to be made between relaxed and contracted tissues, problems such as the following arise: (1) Evaluation of the state of contraction and relaxation of tissues before and during fixation must be provided. (2) Standardization of each step in preparation of tissues for the microscope, such as section thickness, staining and photography must be made. (3) Estimation of the density of photographs or negatives by other than gross subjective means must be included. If contraction does decrease cellular water, osmotic effects of fixatives on cell water content should also be studied.

In the present study, two types of cells were defined not only on grounds of density differences but based on other characteristics as well (Table 17). Quantitative estimates were completed by counting the number of light and dark cells after various treatments (Table 18).

Contraction or relaxation did not change one population of cells into the other as would be predicted if these conditions were responsible for light and dark cells. Evidence that uterine tissues were contracted or relaxed was provided by measurements of tissue lengths before and during fixation. Furthermore, contracted tissues resulted in apparent increase in cross-sectional cell size and greater cell distortion. Similar changes in size and shape of intestinal smooth muscle cells have been observed after contraction (Lane, 1967). Thus, the results from this study are consistent with the observations that contraction leads to smooth muscle cell contortion whereas relaxation produces more rounded cells (Gansler, 1961; Aita *et al.*, 1968a). However, contraction-relaxation cannot account for other structural differences between light and dark cells.

Fixation artifact. Ganote and Moses (1968) found dark cells mainly in the interior of large blocks of liver tissue after immersion fixation. Light and intermediate density cells were confined to the surface of tissue blocks after conditions thought to lead to poor fixation. However, the tissues were mechanically minced in the fixative which may have injured the surface cells. The absence of light and dark cells after *in situ* fixation of liver indicated that they were produced sometime after removal of the tissues from animals. Therefore, light and dark cells were attributed to inadequate fixation.

Light uterine smooth muscle cells were found only after removal of tissues from animals and immersion in glutaraldehyde in this study as was found for liver tissue. Light smooth muscle cells could be cells which were improperly fixed. In this case, one would expect to find the improperly fixed cells on the interior of blocks of tissue. However, I found light myometrial cells distributed randomly among dark cells as neither type of cell was found concentrated in a particular area of the block except at the dissected surface.

The explanation for finding only one type of smooth muscle cell after permanganate fixation may be related to the oxidative nature of this fixative. Permanganate fixation is known to obliterate much of the fine structure of the uterus (Bergman, 1968) and that of other tissues (Hayat, 1970). Therefore, those structures which distinguish light and dark cells in the uterus may be altered or destroyed. Decreased extracellular space, the result of cellular swelling, has been observed in intestinal smooth muscle fixed in permanganate (Henderson *et al.*, 1971). All cells seen after permanganate fixation may correspond to the light cells seen after glutaraldehyde fixation.

Similarly, only one type of cell was found after osmium tetroxide fixation. Osmium tetroxide could also alter or destroy many of the structural features which distinguish light and dark cells seen after primary glutaraldehyde fixation.

Maturity of cells. One might postulate that light cells are newly differentiated or immature smooth muscle cells which are more easily damaged by handling or more sensitive to the effects of immersion fixation. An immature or primitive type of cell has been described for vascular smooth muscle (Scott *et al.*, 1967). These primitive cells were less electron dense than cells considered to be mature and they lacked membrane vesicles. Immature uterine smooth muscle was studied in young ovariectomized rats and compared with estrogen-stimulated and mature pregnant animals in this investigation. There were signs of maturity in the uteri from estrogen-stimulated and pregnant rats as indicated by the increased cell size in comparison with ovariectomized rat tissue which supports the findings of others (Laguens and Lagrutta, 1964; Bergman, 1968). However, dark cells predominated in tissues from both immature and mature uteri. The presence of light cells was not related to the functional state of the uterus or to the maturity of the cells. Cells described as immature cells in vascular smooth muscle (Scott *et al.*, 1967) could correspond to light cells found in the uterus.

2. *Injury as Responsible for Light Cell Production*

Besides differing in electron density, light uterine smooth muscle cells varied greatly from the structure of dark cells (see Table 15). Gansler (1960, 1961) has reported similar differences for uterine and intestinal smooth muscle. Ganote and Moses (1968) have also noted differences in light and dark liver cells. These structural differences suggest that light cells are distinct metabolically from dark cells.

Light cells contained mitochondria which were usually in the orthodox configuration as described by Hackenbrock (1966) for isolated mitochondria while condensed forms were found in dark cells. If mitochondrial structure can be used as an indicator of cellular activity, then light cells were in a metabolic state different from that of dark cells at the time of fixation.

Light cells also had a swollen nuclear envelope, swollen endoplasmic reticulum and were increased in size as also noted by Aita *et al.* (1968a). These changes may be indicative of cellular swelling. Light cells also lacked membrane continuity which suggests the absence of membrane integrity and loss of permeability control. Dark cells had membrane vesicles whereas light cells had few or none. This may denote loss of metabolically dependent mechanisms for controlling cell volume (see Chapter 2). Gansler (1961) also found that vesicles were absent from light smooth muscle cells.

After ATP depletion all smooth muscle cells had characteristics of light cells. Probably light cells seen in tissues after immersion fixation did not contain ATP when fixed for electron microscopy.

Light cells and the structural changes observed in smooth muscle after metabolic or mechanical injury are identical to those described for toad bladder epithelial cells after similar treatments (Saladino *et al.*, 1969; Croker *et al.*, 1970; Saladino *et al.*, 1971). These investigators found that treatment of epithelial cells with detergents or metabolic inhibitors resulted in a sequence of changes which eventually lead to cell death. These changes included swollen endoplasmic reticulum and swollen nuclear envelope, orthodox mitochondrial configurations, loss of membrane structure and decreased cell density. These characteristics are identical to those of light myometrial cells described in this study. Furthermore, similar ultrastructure was observed in the final stages of cell death after injury of various other cells in tissues studied by Trump and Ericsson (1965). Therefore, light uterine smooth muscle cells seen after glutaraldehyde fixation are probably cells injured or dead at the time of fixation.

Permanganate or osmium tetroxide may injure all the cells during fixation.

The electron density of smooth muscle cells could be related to their degree of hydration as proposed by Somlyo *et al.* (1971) and Dohrman (1970) for liver cells. Somlyo and coworkers found increased electron density of some smooth muscle cells after bathing tissues in hypertonic solutions. They do not describe any characteristics other than electron density. If other differences were not present, they were probably dealing with a different phenomenon. However, hypertonic treatment would be expected to decrease cellular water content and thereby may increase the electron density. Light cells found after hypertonic treatment could be cells which failed to respond to osmotic changes because they have lost their semipermeability. Therefore, light cells are probably cells which contained an increased water content due to the loss of volume control. There was a close correspondence between the increased number of light cells and the increased water content of tissues following metabolic inhibition noted in this study. Furthermore, cells without vesicles should be unable to maintain their volume according to the vesicular hypothesis (see Chapter 2). Necrosis of epithelial cells produced by metabolic inhibition with iodoacetic acid resulted in similar density changes in the cells accompanied by volume changes (Saladino *et al.*, 1970).

3. *Conclusion*

Light uterine smooth muscle cells seen after *in vitro* fixation with glutaraldehyde are believed to be the result of mechanical injury to some cells prior to fixation. The injury probably occurs during removal of the tissue from the animal and subsequent handling before immersion in the fixative. Any cell which was sufficiently injured may acquire the appearance of a light cell from loss of metabolism. The density of the light cells could be related to increased water content from loss of metabolically-dependent mechanisms for controlling cell volume. The light cells have no membrane vesicles which are believed to be sites of volume control in uterine smooth muscle (see Chapter 2).

CHAPTER 5

REVIEW AND CONCLUSIONS

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A. Objectives

This chapter will be devoted to a brief recapitulation of the previous chapters.

B. Vesicular Transport by Smooth Muscle

Evidence has been presented which indicated that two mechanisms control cell volume in smooth muscle of the rat uterus. (Daniel and Robinson, 1971a,b,c; Rangachari *et al.*, 1972). One mechanism was ouabain-sensitive, ATP-dependent and it had other characteristics of the classical Na^+-K^+-ATPase found in other tissues (see Skou, 1965). The other mechanism, according to the above investigators, had the following characteristics: (1) It was ATP-dependent. (2) It did not require K^+ in the external solution and it was not sensitive to ouabain. (3) It required Ca^{++}. (4) It had no specificity for Na^+. These characteristics were similar to those of a mechanochemical system proposed for kidney cells (Rorive and Kleinzeller, 1972). In uterine smooth muscle, the membrane vesicles were proposed as sites for volume control (Daniel and Robinson, 1971c).

To test the vesicular transport hypothesis (Chapter 2), pieces of rat myometrium were incubated in Krebs-Ringer solution with metabolism or transport inhibitors. Tissues from the opposite horn were used as the untreated controls. At various times, tissues were removed from the incubation solution and some pieces analyzed for Na^+, K^+, water and ATP contents; other pieces of tissue were fixed and examined in the electron microscope.

Metabolic inhibition with IAA + DNP resulted in a rapid decrease in ATP content of tissues. Swelling and loss of vesicles, gain in Na^+, loss of K^+ and gain in water followed the decrease in ATP content of tissues. The sequence of these changes correlated well with the membrane vesicular model. The simultaneous decrease in vesicular number with decrease in ATP contents was interpreted as an indication of ATP dependence. Na^+ release within the vesicles was considered to be responsible for vesicle swelling.

Treatment of tissues with IAA alone or ethacrynic acid resulted in effects similar to those obtained with the combined use of IAA + DNP. The changes in vesicular number were thought to be associated with the reduction in tissue [ATP].

Ouabain did not change either the ATP content of the tissues or the number of vesicles even after prolonged periods of incubation. These results indicated the insensitivity of the vesicular system to inhibition of Na^+-K^+-ATPase in fresh tissues.

The vesicle number was reduced in tissues made Na^+-

rich by overnight incubation in K^+-free solutions at 5°C. This was believed to be due to the effect of cooling on vesicle formation and discharge. Rewarming the Na^+-rich tissues caused a loss of Na^+ with water and increased the number of vesicles under conditions thought to inhibit Na^+-K^+-ATPase. The vesicle number did not increase in Na^+-rich tissue rewarmed under conditions of maximum Na^+-K^+-ATPase activity. These results may indicate a reciprocal relationship between activity of the vesicular system and that of Na^+-K^+-ATPase. Ca^{++} was necessary for both the loss of Na^+ with water and the increase in number of vesicles observed upon rewarming of Na^+-rich tissues. This evidence may indicate the presence of a contractile mechanism associated with vesicle formation or discharge.

The vesicle number was also reduced in tissues made K^+-rich in Na^+-free, K^+-enriched solutions in the cold. K^+-rich tissues lost water and K^+ and the vesicle number increased when these tissues were rewarmed. This indicated that the vesicular system was not specifically stimulated by Na^+.

Thus, there is good correlation between properties of the volume pump and properties of membrane vesicles of rat uterine smooth muscle. Both systems are: (1) ATP-dependent; (2) insensitive to ouabain; (3) Ca^{++}-dependent; (4) not specific for Na^+. This evidence supports the membrane vesicles as sites for volume control in rat uterine smooth muscle.

C. Evaluation of the Pyroantimonate Technique

Potassium pyroantimonate has been used as an electron microscopy tool for the localization of tissue Na^+. The technique was evaluated (Chapter 3) because of its possible usefulness in localizing Na^+-transport sites.

Fresh, Na^+-rich and Na^+-poor rat uterine tissues were fixed in OsO_4 with or without added pyroantimonate; then washed and dehydrated as for electron microscopy. At each step the tissues and solutions were analyzed spectrophotometrically for Na^+, K^+, Ca^{++}, and Mg^{++} (i.e., cations which precipitate with pyroantimonate). Pyroantimonate was shown not to interfere with these analyses.

Tissues fixed in the presence of potassium pyroantimonate contained very little of their original Na^+ (ca. 10%) but retained increased K^+ and much of their Ca^{++} and Mg^{++}. ^{22}Na and ^{124}Sb-pyroantimonate were used to confirm these results. There was no correlation between the initial or final Na^+ content of tissues and either the amount of electron-dense precipitate or the ^{124}Sb-pyroantimonate content. The distribution of precipitate was a function of the fixative used. The pyroantimonate was thought to be mainly extracellular in glutaraldehyde-fixed tissue. Fifty per cent of the precipitates seen in electron micrographs of tissues fixed in OsO_4 were estimated to be from the added potassium pyroantimonate, and form during dehydration due to the insolubility of this compound in dehydrating solutions. Twenty-five per cent of the precipitates were thought to be pyroantimonate associated with cations which remained in the tissues after fixation. The remaining twenty-five per cent or more of the precipitates were probably due to the binding of pyroantimonate to other tissue constituents. There were no, or inconsistent, precipitates found associated with the membrane vesicles of smooth muscle cells.

In light of this study, pyroantimonate can not be used to demonstrate Na^+-transport sites in uterine smooth muscle. Furthermore, unless other tissues can be shown to react differently to pyroantimonate, the technique should be abandoned and results obtained with it should be discarded.

D. Light and Dark Smooth Muscle Cells.

Two types of smooth muscle cells (termed light and dark cells) were distinguished in the same tissue preparation following *in vitro* fixation in glutaraldehyde. The two types of cells were defined and factors which might affect their number were studied.

The light cells accounted for approximately 4% of the total cells observed in tissues removed from animals and incubated in Krebs-Ringer solution prior to immersion in glutaraldehyde. Relaxation, contraction or the hormonal state of the uteri did not change the relative number of light to dark cells. Fixation in either OsO_4 or potassium permanganate resulted in cells with characteristics similar to the light cells found after *in vitro* glutaraldehyde fixation. Only dark cells were found in tissues after *in situ* fixation with glutaraldehyde. Increased numbers of light cells were found after both chemical or mechanical injury.

Mechanical injury to some cells during removal of the tissues from animals, with subsequent loss of ATP and mechanisms for controlling cell volume, was considered responsible for producing light cells.

Bibliography

Aita, M., Conti, G., Laszt, L. and Mandi, B. (1968a). Sur l'existence de deux sortes de cellules musculaires lisses dans la paroi carotidienne de bovides. Angiologica, Basel 5: 310-321.

Aita, M., Conti, G., Laszt, L. and Mandi, B. (1968b). Etudes immunochimiques et immunohistochimiques des proteines contractiles de la paroi vasculaire de bovides. Angiologica, Basel 5: 322-332.

Albers, R.W., Koval, G.J. and Siegel, G.J. (1968). Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. Mol. Pharmacol. 4: 324-336.

Allen, S.R. and Carstens, L.A. (1966). Electron microscopic alterations in the liver of chickens fed toxic fat. Lab. Invest. 15: 970-979.

Allen, J.C. and Daniel, E.E. (1964). The correlation of inhibition of uphill ion movements and initiation of contracture in intact rat myometrium with inhibition of Mg and Na + K-activated ATPase in myometrial homogenates. Life Sci. 3: 943-952.

Amakawa, T., Mizuhira, V., Uchida, K., Shiina, S., and Tsuji, K. (1968). Evaluation of the sodium-ion detection method Na-electron microscopic autoradiography. J. Elec. Micros. 17: 267-268.

Baker, P.F. and Shaw, T.I. (1965). A comparison of the phosphorus metabolism of intact squid nerve with that of the isolated axoplasm and sheath. J. Physiol. 180: 424-438.

Banks, B.E.C. and Vernon, C.A. (1970). Reassessment of the role of ATP *in vivo*. J. Theor. Biol. 29: 301-326.

Batra, S. and Daniel, E.E. (1970). Dissociation of the downhill calcium movements from sodium and potassium movements in rat uterus. Can. J. Physiol. Pharmacol. 48: 768-773.

Beament, J.W.L. (1964). The active transport and passive movement of water in insects. Advan. Insect Physiol. 2: 67-130.

Bennett, H.S. (1956). The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. J. Biophys. and Biochem. Cytol. 2, Suppl: 99-103.

Bergman, R.A. (1958). Intercellular bridges in ureteral smooth muscle. John Hopkins Hosp. Bull. 102: 195-202.

Bergman, R.A. (1968). Uterine smooth muscle fibers in castrate and estrogen-treated rats. J. Cell Biol. 36: 639-648.

Bettex-Golland, M. and Luscher, E.F. (1965). Thrombosthenin, the contractile protein from blood platelets and its relation to contractile proteins. Adv. Protein Chem. 20: 1-32.

Boyle, P.J. and Conway, E.J. (1941). Potassium accumulation in muscle and associated changes. J. Physiol. 100: 1-63.

Bruns, R.R. and Palade, G.E. (1968a). Studies on blood capillaries. I. General organization of blood capillaries in muscle. J. Cell Biol. 37: 244-276.

Bruns, R.R. and Palade, G.E. (1968b). Studies on blood capillaries. II. Transport of ferritin molecules across the wall of muscle capillaries. J. Cell Biol. 37: 277-299.

Bulger, R.E. (1969). Use of potassium pyroantimonate in the localization of sodium ions in rat kidney tissues. J. Cell Biol. 40: 79-94.

Burnstock, G. (1958). Action of adrenaline on excitability and membrane potential in the taenai coli of the guinea-pig and the effect of DNP on this action and on the action of acetylcholine. J. Physiol. 143: 183-194.

Burnstock, G. (1970). Structure of smooth muscle and its innervation. Edited by Bulbring, E., Brading, A., Jones, A.W., Tomita, T. Arnold, London, pp. 1-69.

Caesar, R., Edwards, G.A., and Ruska, H. (1957). Architecture and nerve supply of mammalian smooth muscle tissue. J. Biophys. Biochem. Cytol. 3: 867-878.

Caldwell, P.C. and Keynes, R.D. (1959). The effect of ouabain on efflux of sodium from squid giant axon. J. Physiol. 148: 8P.

Caldwell, P.C. (1960a). The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. J. Physiol. 152: 545-560.

Caldwell, P.C., Hodgkin, A.L., Keynes, R.D., and Shaw, T.I. (1960b). The effects of injecting "energy-rich" phosphate compounds on the active transport of ions in the giant axons of Logligo. J. Physiol. 152: 561-590.

Casteels, R. (1969). Ion content and ion fluxes in the smooth muscle cells of the longitudinal layer of the guinea pig's vas deferens. Pflugers Arch. 313: 95 - 105.

Charnock, J.S. and Post, R.L. (1963a). Studies on the mechanism of cation transport. I. The preparation and properties of a cation stimulated adenosine-triphosphatase from guinea pig kidney cortex. Aust. J. Exp. Biol. Med. Sci. 41: 547 - 560.

Charnock, J.S., Post, R.L. and Rosenthal, A.S. (1963b). Studies on the mechanism of cation transport. II. A phosphorylated intermediate in the cation stimulated enzymatic hydrolysis of adenosine-triphosphate. Aust. J. Exp. Biol. Med. Sci. 41: 675 - 686.

Charnock, J.S. and Opit, L.J. (1968). Membrane metabolism and ion transport in the biological basis of medicine, Vol. I. pp. 69 - 104. Edited by Bittar, E.E. and Bittar, N. Academic Press.

Clark, M.A. and Ackerman, G.A. (1971a). Alteration of nuclear and nucleolar pyroantimonate-osmium reactivity by glutaraldehyde fixation. J. Histochem. Cytochem. 19: 388 - 390.

Clark, M.A. and Ackerman, G.A. (1971b). A histochemical evaluation of the pyroantimonate-osmium reaction. J. Histochem. Cytochem. 19: 727 - 737.

Conti, G., Haenni, B. and Roviller Ch. (1964). Structure et ultrastructure de la cellule musculaire lisse de la paroi carotidienne a l'etat de repos et a l'etat de contraction. Angiologica, Basel 1: 119 - 140.

Conti, G., Modis, L. and Gotzos, V. (1972). Etudes au microscope polarisant des cellules musculaires lisses de la carotide de bovides. Acta. Anat. 81: 119 - 126.

Cope, F.W. (1967). NMR evidence for complexing of Na^+ in muscle, kidney, brain, by actomyosin. The relation of cellular complexing of Na^+ to water structure and to transport kinetics. J. Gen. Physiol. 50: 1353 - 1575.

Cope, F.W. (1970). Spin-echo nuclear magnetic resonance evidence for complexing of sodium ions in muscle, brain and kidney. Biophys. J. 10: 843 - 858.

Croker, B.P., Saladino, A.J. and Trump, B.F. (1970). Ion movements in cell injury: relationship between energy metabolism and the pathogenesis of lethal cell injury in the toad bladder. Am. J. Path. 59: 247 - 278.

Csapo, A. and Gergely, J. (1950). Energetics of uterine muscle contraction. Nature 166: 1078 - 1079.

Daniel, E.E., Sehder, H. and Robinson, K. (1962). Mechanisms for activation of smooth muscle. Physiol. Rev. 42: 228 - 264.

Daniel, E.E. (1963a). Potassium movements in rat uterus studied *in vitro* I. Effects of temperature. Can. J. Biochem. Physiol. 41: 2065 - 2084.

Daniel, E.E. (1963b). Potassium movements in rat uterus studies *in vitro* II. Effects of metabolic inhibitors, ouabain and latered potassium concentration. Can. J. Biochem. Physiol. 41: 2085 - 2105.

Daniel, E.E. (1964). Interconnection between active transport and contracture in uterine tissue. Can. J. Physiol. Pharmacol. 42: 453 - 495.

Daniel, E.E. and Robinson, K. (1960). Relation of sodium secretion to metabolism in isolated sodium-rich uterine segments. J. Physiol. 154: 445 - 460.

Daniel, E.E., Robinson, K., Kidwai, A.M., Wolowyk, M.W., Taylor, G.S. and Paton, D.M. (1969). The sodium pump in smooth muscle. Proc. Symposium on the Physiology and Pharmacology of Vascular Neuroeffector Systems, S. Karger, Basel.

Daniel, E.E. and Robinson, K. (1970). Sodium exchange and net movement in rat uteri at 25°C. Can. J. Physiol. Pharmacol. 48: 598 - 624.

Daniel, E.E. and Robinson, K. (1971a). Effects of inhibitors of active transport on ^{22}Na and ^{42}K movements and on nucleotide levels in rat uteri at 25°C. Can. J. Physiol. Pharmacol. 49: 178 - 204.

Daniel, E.E. and Robinson, K. (1971b). Effects of inhibitors of metabolism on adenine nucleotides and on ^{22}Na and ^{42}K and net movements in rat uteri at 25°C. Can. J. Physiol. Pharmacol. 49: 205 - 239.

Daniel, E.E. and Robinson, K. (1971c) The effects of temperature on sodium movements in rat uteri and a model for control of their ion content. Can. J. Physiol. Pharmacol. 49: 240 - 262.

Daniel, E.E., Kidwai, A.M., Robinson, K., Freeman, D. and Fair, S. (1971d). The mechanisms by which ethacrynic acid affects ion content, ion fluxes, volume and energy supply in the rat uterus. J. Pharmac. Exp. Therap. 176: 563 - 579.

Dean, R.B. (1941). Theories of electrolyte equilibrium in muscle. Biol. Symp. 3: 331 - 348.

Desmedt, J.E. (1953). Electrical activity and intracellular sodium concentration in frog muscle. J. Physiol. 121: 191 - 205.

Devine, D.W., Simpson, F.O. and Bertaud, W.S. (1971). Surface features of smooth muscle cells from the mesenteric artery and vas deferens. J. Cell. Sci. 8: 427 - 443.

Devine, C.E. and Somlyo, A.P. (1970). Ultrastructure of vascular smooth muscle (VSM) studied with lanthanum. Fed. Proc. 29: 455 (Abstr.)

Dohrmann, G.J. (1970). Dark and light epithelial cells in the choroid plexeis of mammals. J. Ultrastruc. Res. 32" 268 - 273.

Duggan, D.E. and Noll, R.M. (1966). A biochemical basis for the inactivity of ethacrynic acid in the rat. Biochim. Biophys. Acta. 121: 162 - 164.

Dunham, E.T. and Glynn, I.M. (1961). Adenosine-triphosphatase activity and the active movements of alkali metal ions. J. Physiol. (London) 156: 274 - 293.

Dunn, M.J. (1970). The effects of transport inhibitors on sodium outflux in red blood cells: Evidence for exchange diffusion. J. Clin. Invest. 49: 1804 - 1814.

Edelhauser, H.F. and Siegesmund, K.A. (1968). The localization of sodium in the teleost cornea. Invest. Ophth. 7: 147 - 155.

Fenn, W.O. (1938). Factors affecting the loss of potassium from stimulated muscles. Am. J. Physiol. 124: 213 - 229.

Gabella, G. (1971). Caveolae intracellulares and sarcoplasmic reticulum in smooth muscle. J. Cell. Sci. 8: 601 - 609.

Gabella, G. (1972). The arrangement of sarcoplasmic reticulum in smooth muscle. Experientia 28: 948 - 949.

Ganote, C.E. and Moses, H.L. (1968). Light and dark cells as artifacts of liver fixation. Lab. Invest. 18: 740 - 745.

Gansler, H. (1960). Phasenkontrast-und elektronenmikroskopische Untersuchungen zur Morphologic und Funktion der glätten Muskulatur. Z. Zelltorsch 52: 60 - 92.

Gansler, H. (1961). Structur und Funktion der glatten Muskulatur. II. Licht-und elektronenmikroskopische Befunde an Hohlorganen von Ratten, Meerschweinchen und Mensch. A. Zellforsch. Mikrosk. Anat. 55: 724 - 762.

Gardos, (1954). Accumulation of potassium ions in human blood cells. Acta. Physiol. Acad. Sci. Hung. 6: 191 - 199.

Garrahan, P.J. and Glynn, I.M. (1967a). The behaviour of the sodium pump in red cells in the absence of external potassium. J. Physiol. 192: 159 - 174.

Garrahan, P.J. and Glynn, I.M. (1967b). Factors affecting the relative magnitudes of the sodium: Potassium and sodium: Sodium exchanges catalysed by the sodium pump. J. Physiol. 192: 189 - 216.

Ghidoni, J.J. (1967). Light and electron microscopic study of primate liver 36 to 48 hours after high doses of 32-million electron-volt protons. Lab. Invest. 16: 268 - 286.

Gibbons, J.R. and Gristone, A.V. (1960). On flagellar structure in certain flagellates. J. Biophys. Biochem. Cytol. 7: 697 - 710.

Glynn, I.M. (1957). The action of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol. 136: 148 - 173.

Goodford, P.J. (1968). Distribution and exchange of electrolytes in intestinal smooth muscle. In: Handbook of Physiology. Alimentary Canal IV. pp. 1743 - 1766. Edited by C.F. Code, Amer. Physiol. Soc., Washington, D.C.

Goodford, P.J., Johnson, F.R., Krasucki, Z. and Daniel, V. (1968). Transport of sodium in smooth muscle cells. J. Physiol. 194: 77P - 78P.

Goodford, P.J. (1970). Ionic interactions in smooth muscle. pp. 100 - 121. In: Smooth Muscle. Edited by Bulbring, E., Brading, A.F., Jones, A.W., Tomita, T., Arnold, London.

Goodford, P.J. and Wolowyk, M.W. (1972). Localization of cation interactions in the smooth muscle of the guinea pig taenia coli. J. Physiol. 224: 521 - 535.

Green, D.E. (1970). The conformational basis of energy transductions in biological systems. Proc. Natl. Acad. Sci. 67: 544 - 549.

Hackenbrock, C.R. (1966). Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J. Cell. Biol. 30: 269 - 297.

Hardin, J.H., Spicer, S.S., Greene, W.B. (1969). The paranucleolar structure, accessory body of Cajal, sex chromatin, and related structures in nuclei of rat trigeminal neurons: A cytochemical and ultrastructural study. Anat. Rec. 164: 403 - 432.

Hardin, J.H. and Spicer, S.S. (1970). An ultrastructural study of human eosinophil granules: Maturational stages and pyroantimonate reactive cation. Am. J. Anat. 128: 283 - 310.

Harris, E.J. and Maizels, M. (1952). Distribution of ions in suspensions of human erthrocytes. J. Physiol. 118: 40 - 53.

Hartmann, J.F. (1966). High sodium content of cortical astrocytes. Arch. Neurol. 15: 633 - 641.

Hayat, M.A. (1970). Principles and techniques of electron microscopy. Van Nostrand Reinhold Co., New York.

Hazelwood, C.F., Nichols, B.L. and Chamberlain, N.F. (1969). Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle. Nature 222: 747 - 750.

Henderson, R.M., Duchon, G., Daniel, E.E. (1971). Cell contacts in duodenal smooth muscle layers. Am. J. Physiol. 221: 564 - 574.

Henrikson, R.C. (1970). Ultrastructure of ovine fuminal epithelium and localization of sodium in the tissue. J. Ultrastruc. Res. 30: 385 - 401.

Heppel, L.A. (1940). The diffusion of radioactive sodium into the muscles of potassium-deprived rats. Am. J. Physiol. 128: 449 - 454.

Herdson, P.B., Garvin, P.J. and Jennings, R.B. (1964). Reversible biological and fine structural changes produced in rat liver by a thiohydantoin compound. Lab. Invest. 13: 1014 - 1031.

Hinke, J.A.M. and McLaughlin, (1967). Release of bound sodium in single muscle fibers. Can. J. Physiol. Pharmacol. 45: 655 - 667.

Hirsch, S. (1955). Uber die morphologischen Merkmale der Vasocon-stricktion beim Menschen, zugleich ein Beitrag zum problem der Function der glatten Muskelfasen. Acta. Med. Scand. 152: 379 - 390.

Hodgkin, A.L. and Katz, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol 108: 37 - 77.

Hodgkin, A.L. and Keynes, R.D. (1955). Active transport of cations in giant axons from Sepia and Loligo. J. Physiol. 128: 28 - 60.

Hodgman, C.D. (ed.). (1943). "Handbook of Chemistry and Physics", 27th ed. The Chemical Rubber Company. Cleveland, Ohio, pp. 456 - 457.

Hoffman, J.F. (1960). The link between metabolism and the active transport of Na in human red cell ghosts. Fed. Proc. 19: 127.

Hoffman, J.F. (1966). The red cell membrane and the transport of sodium and potassium. Am. J. Med, 41: 666 - 680.

Hoffman, J.F. and Kregenow, F.M. (1966). The characterization of new energy depent cation transport processes in red blood cells. Ann. N.Y. Acad. Sci. 137: 566 - 576.

Hoffmann-Berling, H. (1956). Das Kontraktile eiweiss undifferenzierter zellen. Biochim. Biophys. Acta. 19: 453 - 463.

Hokin, L.E. (1969). On the molecular characterization of the sodium-potassium transport adenosine-triphosphatase. J. Gen. Physiol. 54: 327s - 342s.

Horowicz, P. (1965). Sodium movements in frog's sartorium muscle. Acta. Physiol. Acad. Sci. Hung. 26: suppl. 14 - 15.

Jaeger, J. (1964). Variations in the morphological substrate of the smooth muscle cells of the pregnant and non-pregnant human uterus. Uterine contractility, symposium in Wurzburg. Bibl. Gynaec., Fasc. 42: 13 - 28.

Jones, V.D. and Landon, E.J. (1967). The effect of ouabain, meralluride and ethacrynic acid on respiration and glycolysis in kidney slices. Biochem. Pharmacol. 16: 2163 - 2169.

Kahllen, D.A. and Koch, H.J. (1967). Methodische untersuchungen fur quantitative mikrobestimmung von ATP in biologischen material mit dem firefly-enzym system. Z. Klin, Chem. Klin, Biochem. 5: 299 - 304.

Kao, C.Y. and Nishiyama, A. (1969). Ion concentrations and membrane potentials of myometrium during recovery from cold. Am. J. Physiol. 27: 525 - 531.

Kaye, G.I. and Donn, A. (1965). Studies on the cornea. IV. Some effects of ouabain on pinocytosis and stromal thickness in the rabbit cornea. Invest. Ophtha. 4: 844 - 852.

Keynes, R.D. (1966). Exchange diffusion of sodium in frog muscle. J. Physiol. 184: 31P - 32P.

Kierszenbaum, A.L., Libanati, C.M. and Tandler, C.J. (1971). The distribution of inorganic cations in mouse testis. Electron microscope and microprobe analysis. J. Cell. Biol. 48: 314 - 323.

Klein, R.L., Horton, C.R. and Thureson-Klein, A. (1970). Studies on nuclear amino acid transport and cation content in embryonic myocardium of the chick. Am. J. Cardiol. 25: 300 - 310.

Klein, R.L., Yen, S. and Thureson-Klein, A. (1972). Critique on the K-pyroantimonate method for semi-quantitative estimates of cations in conjunction with electron microscopy. J. Histochem. Cytochem. 20: 65 - 78.

Kleinzeller, A. and Knotkova, A. (1964). The effect of ouabain on the electrolyte and water transport in kidney cortex and liver slices. J. Physiol. 175: 172 - 192.

Komnick, H. (1962). Elektronen-mikroskopische lokalisation von Na^+ und Cl^- in Zellen und Geweben. Protoplasma 55: 414 - 418.

Komnick, H. und Komnick, U. (1963). Elektronenmikroskopische untersuchungen zur funktionellen morphologie des ionentransportes in der Salzdruse von Larus argentatus. V. Teil: Experimenteller Nachweis der Transportwege. Z. Zellforsch 60: 163 - 203.

Komnick, H. and Bierther, M. (1969). Zur histochemischen Ionenlokalization mit Hilfe der Elektronenmikroskopie. Histochemie 18: 337 - 362..

Krames, B. and Page, E. (1968). Effects of electron-microscopic fixatives on cell membranes of the perfused rat heart. Biochem. Biophys. Acta. 150: 24 - 31.

Lafontaine, J.G. and Allard, C. (1964). A light and electron microscope study of the morphological changes induced in rat liver cells by the A20 dye 2-ME-DAB. J. Cell. Biol. 22: 143 - 172.

Laguens, R. and Lagrutta, J. (1964). Fine structure of human uterine muscle in pregnancy. Am. J. Obst. Gynec. 89: 1040 - 1048.

Lane, B.P. (1965). Alterations in the cytologic detail of intestinal smooth muscle cells in various stages of contraction. J. Cell. Biol 27: 199 - 213.

Lane, B.P. (1967). Localization of products of ATP hydrolysis in mammalian smooth muscle cells, J. Cell. Biol. 34: 713 - 720.

Lane, B.P. and Martin, E. (1969). Electron probe analysis of cationic species in pyroantimonate precipitates in Epon embedded tissue. J. Histochem. Cytochem. 17: 102 - 106.

Le Blanc, G. and Erlij, D. (1969). Effects of ethacrynic acid on Na^+ fluxes in frog sartorius muscle. Biochem. Biophys. Acta. 173: 149 - 151.

Lee, J.C., Johanson, L. and Hopper, J. (1967). Compensatory renal hypertrophy: Ultrastructural changes and histochemical localization of sodium ion in proximal tubulen. Am. J. Path. 50: 50a.

Legato, M.J. and Langer, G.A. (1969). The subcellular localization of calcium ion in mammalian myocardium. J. Cell. Biol. 41: 401 - 423.

Lennep, E.W. van (1968). Electron microscopic histochemical studies on salt-excreting glands in elasmobranchs and marine catfish. J. Ultrastruc. Res. 25: 94 - 108.

Lennep, E.W. van and Komnick, H. (1971). Histochemical demonstration of sodium and chloride in the frog epidermis. Cytobiol. 1: 137 - 151.

Lin, S. and Cohen, H.P. (1968). Measurement of adenosine-triphosphate content of crayfish stretch receptor cell preparations. Anal. Biochem. 24: 531 - 540.

Ling, G.N. (1962). A physical theory of the living state: The association-induction hypothesis. Blaisdell, New York.

Ling, G.N. (1966). Cell membrane and cell permeability. Ann. N.Y. Acad. Sci. 137: 837 - 859.

Ling, G.N. (1969). A new model for the living cell: A summary of the theory and recent experimental evidence in its support. Int'l. Rev. Cytol. 26: 1 - 61.

Lubowitz, H. and Whittam, R. (1969). Ion movements in human red cells independent of the sodium pump. J. Physiol. 202: 111 - 131.

Macallum, A.B. (1905). On the distribution of potassium in animal and vegetable cells. J. Physiol. 32: 95 - 128.

MacKnight, A.D.C. (1968). Water and electrolyte contents of rat renal cortical slices incubated in potassium-free media and media containing ouabain. Biochim. Biophys. Acta. 150: 263 - 270.

MacKnight, A.D.C. (1969). The effects of ethacrynic acid on the electrolyte and water contents of rat renal cortical clices. Biochim. Biophys. Acta. 173: 223 - 233.

Maizels, M. and Whittaker, N. (1940). Diluents for stored blood. Lancet 1: 590 - 593.

Maizels, M. (1951). Factors in the active transport of cations. J. Physiol. 112: 59 - 83.

Marchesi, S.L., Steers, E., Marchesi, V.T. and Tillack, T.W. (1969). Physical and chemical properties of a protein isolated from red cell membranes. Biochemistry 9: 50 - 57.

Mark, J.S.T. (1956). An electron microscope study of uterine smooth muscle. Anat. Rec. 125: 473 - 493.

McGill, C. (1909). Structure of smooth muscle in the resting and in the contracted condition. Am. J. Anat. 9: 493 - 545.

Meigs, E.B. and Atwood, W.G. (1916). The reactions of striated muscle to potassium chloride solutions. Am. J. Physiol. 40: 30 - 42.

Millonig, G. (1962). In "Electron Microscopy" (Proc. 5th Intern. Congr. Electron Microscopy), Vol. 2, p. 8. Academic Press, New York.

Moawad, A.H. and Daniel, E.E. (1971). Total contents and net movements of magnesium in the rat uterus. Am. J. Physiol. 220: 75 - 82.

Mullins, L.J. and Frumento, A.S. (1963). The concentration dependence of sodium efflux from muscle. J. Gen. Physiol. 46: 629 - 654.

Needham, D.M. and Shoenberg, C.F. (1967). Biochemistry of the myometrium. In Cellular biology of the uterus. Edited by Wynn, R.W. Appleton-Century Crofts. New York.

Neifakh, S.A., Avramov, J.A., Gaitskhoki, V.S., Kazaliova, T.B., Monakhov, N.K., Repin, V.S., Turovski, V.S. and Vassiletz, I.M. (1965). Mechanism of the controlling function of mitochondria. Biochim. Biophys. Acta. 100: 329 - 343.

Nemetschek-Gansler, H. (1967). Ultrastructure of the myometrium. pp. 353 - 385. In Cellular biology of the uterus. Edited by Wynn, R.M. Appleton-Crofts. New York.

Nobel, P.S. and Murakami, S. (1967). Accumulation of ions by spinach chloroplasts. J. Cell. Biol. 30: 209 - 212.

Nolte, A. (1966). Elektronenmikroskopische untersuchungen zum Natrium und chloridionentransport in der proximalen tubulus zell der Ratten-niere. Zeit. Zellforsch. 72: 562 - 573.

Ochi, J. (1968). Elekronenmikroskopischer Nachweis der Natriumionen in den Schweissdrusen der Rattenfussohle. Histochemie 14: 300 - 307.

Opit, L.J. and Charnock, J.S. (1965). A molecular model for a sodium pump. Nature 208: 471 - 475.

Organ, A.E., Boves, E.C., Jahn, T.L., Wigg, D. and Fonseca, J.R. (1968). The mechanism of the nephridial apparatus of Paramecium multimicronucleatum. J. Cell. Biol. 37: 139 - 145.

Papadimitriou, J.M. (1965). Electron micrographic features of acute murine reovirus hepatitus. Am. J. Pathol. 47: 565 - 585.

Penniston, J.T. and Green, D.E. (1968). The conformational basis of energy transformations in membrane systems. IV. Energized states of pinocytosis in erythrocyte ghosts. Arch. Biochem. Biophys. 128: 339 - 350.

Sen, A.K. and Post, R.L. (1964). Stoichiometry and localization of adenosine-triphosphate dependent sodium and potassium transport -n the erythrocyte. J. Biol. Chem. 239: 345 - 352.

Shea, S.M., Karnovsky, M.J. and Bossert, W.H. (1969). Vesicular transport across endothelium: Simulation of a diffusion model. J. Theor. Biol. 24: 30 - 32.

Shiina, S., Mizuhira, V., Uchida, K. and Amakawa, T. (1969). Electron-microscopic study of sodium ion distribution in cardiac ventricle cells. Jap. Circ. J. 33: 601 - 605.

Shiina, S. and Mizuhira, V. (1970a). EM study on distribution of sodium and potassium in cardiac ventricle muscle cells influenced by ouabain. Jap. Circ. J. 34: 793 - 797.

Shiina, S. and Mizuhira, J. (1970b). Subcellular localization of Na^+ and K^+ ions in skeletal muscle. Acta. Histochem. Cytochem. 3: 74 - 79.

Shiina, S., Mizuhira, V., Amakawa, T. and Futaesaku, Y. (1970). An analysis of the histochemical procedure for sodium ion detection. J. Histochem. Cytochem. 18: 644 - 649.

Siegesmund, K.A. and Edelhauser, H.F. (1968). Na^+ localization in cerebellum. Anat. Rec. 160: 517 - 518.

Simpson, F.O. and Devine, C.E. (1966). The fine structure of autonomic neuromuscular contacts in arterioles of sheep renal cortex. J. Anat. 100: 127 - 137.

Sjodin, R.A. and Beauge, L.A. (1968). Strophanthidin-sensitive components of potassium and sodium movements in skeletal muscle as influenced by the internal sodium concentration. J. Gen. Physiol. 52: 389 - 407.

Skou, J.C. (1957). The influence of some cations on an adenosine-triphosphatase from peripheral nerves. Biochem. Biophys. Acta. 23: 394 - 401.

Skou, J.C. (1960). Further investigations of a magnesium requiring sodium activated adenosine-triphosphate. Biochim. Biophys. Acta. 24: 6 - 23.

Skou, J.C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membranes. Physiol. Rev. 45: 596 - 617.

Perkins, D.L. and Jahn, T.L. (1970). Amoeboflagellate transformations and the Gibbs-Donnan ratio. J. Protozool. 17: 168 - 172.

Ponder, E. (1950). Accumulation of potassium by human red cells. J. Gen. Physiol. 33: 745 - 757.

Post, R.L., Merritt, C.R., Kinsolving, C.R. and Albright, C.D. (1960). Membrane adenosine-triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Chem. 235: 1796 - 1802.

Prasad, K. and MacLeod, D.P. (1969). Influence of glucose on the trans-membrane action potential of guinea pig papillary muscle. Circulation Res. 24: 939 - 950.

Prosser, C.L., Burnstock, G. and Kahn, J. (1960). Conduction in smooth muscle: Comparative structural properties. Am. J. Physiol. 199: 545 - 552.

Rangachari, P.S.K. (1972). Metabolic requirements for coupled Na^+-K^+ exchange and spontaneous contractions in the rat myometrium. Ph.D. Thesis, University of Alberta.

Rangachari, P.S.K., Paton, D.M. and Daniel, E.E. (1972). Potassium: ATP ratios in smooth muscle. Biochim. Biophys. Acta. 274: 462 - 465.

Rangachari, P.S.K., Paton, D.M. and Daniel, E.E. (1972). Aerobic and glycolytic support of sodium pumping and contraction in rat myometrium. Am. J. Physiol. 223: 1009 - 1015.

Revel, J.P. and Karnovsky, M.J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J. Cell. Biol. 33: C7.

Reynolds, E.W. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17: 208 - 212.

Rhodin, J.A.G. (1962). Fine structure of fascular walls in mammals with special reference to smooth muscle component. Physiol. Rev. 42: 48 - 81.

Ritch, R. and Philpott, C.W. (1969). Repeating particles associated with an electrolyte-transport membrane. Exp. Cell. Res. 55: 17 - 24.

Rorive, G., Nielsen, R. and Kleinzeller, A. (1972). Effect of pH on the water and electrolyte content of renal cells. Biochim. Biophys. Acta. 266: 376 - 396.

Rorive, G. and Kleinzeller, A. (1972). The effect of ATP and Ca^{+2} on the cell volume in isolated kidney tubules. Biochim. Biophys. Acta. 274: 226 - 239.

Rosenthal, A.S., Moses, H.L., Ganote, C.E. and Tice, L. (1969). The participation of nucleotide in the formation of phosphatase reaction product: a chemical and electron microscope autoradiographic study. J. Histochem. Cytochem. 17: 839 - 847.

Rostgaard, J. and Barrnett, R.J. (1964). Fine structure localization of nucleoside phosphates in relation to smooth muscle cells and unmyelinated nerves in the small intestine of the rat. J. Ultrastruc. Res. 11: 193 - 207.

Rustad, R.C. (1959). Molecular organization at the surface of Amoebae during pinocytosis. Nature 183: 1058 - 1059.

Saladino, A.J., Bentley, P.J., Trump, B.F. (1969). Ion movements in cell injury: Effects of amphotericin B on ultrastructure and function of the epithelial cells of the toad bladder. Am. J. Path. 54: 421 - 466.

Saladino, A., Hawkins, H.K., Trump, B.F. (1971). Ion movements in cell injury: Effects of the cationic detergent cetylpyridinium chloride on the ultrastructure and function of the toad bladder. Am. J. Path. 64: 271 - 294.

Satir, P. and Gilula, N.B. (1970). The cell junction in a lamellibranch gill ciliated epithelium. Localization of pyroantimonate precipitate. J. Cell. Biol. 47: 468 - 487.

Scharrer, E. (1938). On dark and light cells in the brain and in the liver. Anat. Record 72: 53 - 65.

Schatzmann, H.J. (1953). Herzglykoside als Hemmstoffe fur den aktiven Kalium - und Natrium-Transport durch das Erythrocytenmembran. Helv. Physiol. Acta. 11: 389 - 391.

Schatzmann, H.J. (1962). Lipoprotein nature of red cell ATPase. Nature 196: 677.

Scott, R.F., Jones, R., Daoud, A.A., Zumbo, O. and Coulston, F. (1967). Experimental atherosclerosis in rhesus monkeys. II. Cellular elements of proliferative lesions and possible role of cytoplasmic degeneration in pathogenesis as studied by electron microscopy. Exptl. Mol. Pathol. 7: 34 - 37.

Smith, E.K.M., Farrington, D. and Sydiuk, L. (1972). Observations on the measurement and regulation of the sodium content of rabbit erthrocytes: Evidence for a single sodium pump. Canad. J. Physiol. and Pharmacol. 50: 791 - 797.

Somlyo, A.P., Devine, C.E., Somlyo, A.V. and North, S.R. (1971a). Sarcoplasmic reticulum and the temperature-dependent contraction of smooth muscle in calcium-free solutions. J. Cell. Biol. 51: 722 - 741.

Somlyo, A.P., Somlyo, A.V., Devine, C.E. and Rice, R.V. (1971b). Aggregation of thick filaments into ribbons in mammalian smooth muscle. Nature 231: 243 - 246.

Somlyo, A.P. (1972). Excitation-contraction coupling in vertebrate smooth muscle: Correlation of ultrastructure with function. The Physiologist, 15: 338 - 348.

Spicer, S.S., Hardin, J.H. and Greene, W.B. (1968). Nuclear precipitates in pyroantimonate-osmium-fixed tissues. J. Cell. Biol. 39: 216 - 222.

Spicer, S.S., Greene, W.B., Hardin, J.H. (1969). Ultrastructural localization of acid mucosubstance and antimonate-precipitate in human and rabbit platelets and megakaryocytes. J. Histochem. Cytochem. 17: 781 - 792.

Spicer, S.S. and Swanson, A.A. (1972). Elemental analysis of precipitates formed in nuclei by antimonate-osmium tetroxide fixation. J. Histochem. Cytochem. 20: 518 - 526.

Steiner, J.W. And Baglio, C.M. (1963). Electron microscopy of the cytoplasm of parenchymal liver cells in x-naphthyl-isothiocynate-induced cirrhosis. Lab. Invest. 12: 765 - 790.

Sumi, S.M. (1971). Variations in the location and size of pyroantimonate precipitates in the immature rat cerebral cortex. J. Histochem. Cytochem. 19: 591 - 604.

Sumi, S.M. and Swanson, P.D. (1971). Limitations of the pyroantimonate technique for localization of sodium in isolated cerebral tissues. J. Histochem. Cytochem. 19 605 - 610.

Tandler, C.J. and Kierszenbaum, A.L. (1971). Inorganic cations in rat kidney. Localization with potassium pyroantimonate-perfusion fixation. J. Cell. Biol. 50: 830 - 839.

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Tandler, C.J., Labanati, C.M. and Sanchis, C.A. (1970). The intracellular localization of inorganic cations with potassium pyroantimonate. J. Cell. Biol. 45: 355 - 366.

Tani, E., Ametani, T. and Handa, H. (1969). Sodium localization in the adult brain. II. Triethyltin intoxication and cerebral edema produced by epidural compression. Acta. Neuropath. 14: 151 - 160.

Taylor, G.S., Paton, D.M. and Daniel, E.E. (1970). Characteristics of electrogenic sodium pumping in rat myometrium. J. Gen. Physiol. 56: 360 - 375.

Theron, J.J. (1965). Acute liver injury in ducklings as a result of aflatoxin poisoning. Lab. Invest. 14: 1586 - 1603.

Thomas, R.C. (1972). Electrogenic sodium pump in nerve and muscle cells. Physiol. Rev. 52: 563 - 594.

Thureson-Klein, A. and Klein, R.L. (1971). Cation distribution and cardic jelly in early embryonic heart: A histochemical and electron microscope study. J. Mol. Cell. Cardiol. 2: 31 - 40.

Tisher, C.C., Cirksena, W.J., Arstila, A.U. and Trump, B.F. (1969). Subcellular localization of sodium in normal and injured proximal tubules of the rat kidney. Am. J. Path. 57: 231 - 251.

Tisher, C.C., Weavers, B.A. and Cirksena, W.J. (1972). X-ray microanalysis of pyroantimonate complexes in rat kidney. Am. J. Pathol. 69: 255 - 270.

Torack, R.M. (1969). Sodium demonstration in rat cerebrum following perfusion with hydroxyadipaldehyde-antimonate. Acta. Neuropath. 12: 173 - 182.

Torack, R.M. (1971). The penetration of sodium into the brain following a cisternal injection of sodium chloride with particular emphasis on the area postrema. Z. Zellforsch. 113: 1 - 12.

Torack, R.M. and LaValle, M. (1970). The specificity of the pyroantimonate technique to demonstrate sodium. J. Histochem. Cytochem. 18: 635 - 643.

Tosteson, D.C. and Hoffman, J.F. (1960). Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. 44: 169 - 194.

Trump, B.F. and Ericsson, J.L.E. (1965). Some ultrastructural and biochemical consequences of cell injury. In the inflammatory process. Edited by Zweifach, B.W., Grant, L. and McCluskey, R.T. Acad. Press, New York. pp. 35 - 120.

Ussing, H.H. (1949). Transport across cellular membranes. Physiol. Rev. 49: 127 - 155.

Ussing, H.H. (1954). Active transport of inorganic ions. Symp. Soc. Exp. Biol. 8: 407 - 422.

Vignais, P.V., Vignais, P.M., Rossi, C.S. and Lehninger, A.L. (1963). Restoration of ATP-induced contraction of pretreated mitochondria by "Contractile Protein". Biochim. Biophys. Res. Comm. 11: 307 - 312.

Villegas, J. (1968). Transport of electrolyten in the schwann cell and location of sodium by EM. J. Gen. Physiol. 51: 61s - 71s.

Vinnikov, Y.A. and Koichev, K. (1969). Guinea pig spiral organ during quiet and sound. Nature 223: 641 - 642.

Waalas, O. and Waalas, E. (1950a). The content of adenosinetriphosphate and creating phosphate in uterine muscle of rats and rabbits. Acta. Physiol. Scand. 21: 1 - 17

Waalas, O. and Waalas, E. (1950b). The metabolism of uterine muscle studied with P32. Acta. Physiol. Scand. 21: 18 - 26.

Wells, G.S. and Wolowyk, M.W. (1971). Freeze-etch observations on membrane structure in the smooth muscle of guinea pig taenia coli. J. Physiol. 218: 11 - 13p.

Whittam, R. (1958). Potassium movements and ATP in human red blood cells. J. Physiol. 140: 479 - 497.

Whittam, R. (1962). Asymmetrical stimulation of membrane adenosinetriphosphate in relation to active cation transport. Biochem. J. 84: 110 - 118.

Whittam, R. and Wheeler, K.P. (1970). Transport across cell membranes. Ann. Rev. Physiol. 32: 21 - 60.

Whittembury, G. (1968). Sodium and water transport in kidney proximal tubular cells. J. Gen. Physiol. 51: 303s - 314s.

Whittembury, G. and Fishman, J. (1969). Relation between cell Na extrusion and transtubular absorption in the perfused toad kidney. The effect of K, ouabain and ethacrynic acid. Pfluger's Arch. 307: 138 - 153.

Whittembury, G. and Proverbio, F. (1970). Two modes of Na extrusion in cells from guinea pig kidney cortex slices. Pflugers Arch. 316: 1 - 25.

Wilbrandt, W. (1937). A relation between the permeability of the red cell and its metabolism. Trans. Faraday Soc. 33: 956 - 959.

Willis, J.S. (1968). The interaction of K^+, ouabain and Na^+ on the cation transport and respiration of renal cortical cells of hamsters and ground squirrels. Biochim. Biophys. Acta. 163: 516 - 530.

Wirth, H., Daniel, E.E. and Carrol, P.M. (1970). The effects of adenosine deaminase inhibitor and 2-mercaptoethariol on frozen-thawed uterine horns. Cryobiol. 6: 395 - 400.

Witzleben, C.L. (1966). An electron microscopic study of ferrow sultate induced liver damage. Am. J. Path. 49: 1053 - 1067.

Wood, R.L. (1965). The fine structure of hepatic cells in chronic ethionine poisoning and during recovery. Am. J. Pathol. 46: 307 - 330.

Yamauchi, A. and Burnstock, G. (1969). Post-natal development of smooth muscle cells in the mouse vas deferens. A fine structural study. J. Anat. 104: 1 - 15.

Zadunaisky, J.A. (1966). The localization of sodium in the transverse tubules of skeletal muscle. J. Cell. Biol. 31: 11c - 16c.

Zadunaisky, J.A., Gennaro, J.F., Jr., Bashirelahi, N. and Hilton, M. (1968). Intracellular redistribution of sodium and calcium during stimulation of sodium transport in epithelial cells. J. Gen. physiol. 51: 290s - 302s.

Appendix

The chemicals used in this study with their abbreviations and sources are given below:

Chemical	Abbreviation	Source
Iodoacetate	IAA	Sigma Chemical Co.
Dinitrophenol	DNP	Fisher Scientific Co.
Ethacrynic Acid	EA	Merck Sharp and Dohme
Ouabain		Nutritional Biochemical Corp.
Adenosine triphosphate	ATP	Sigma Chemical Co.
Sodium pyruvate	pyruvate	Sigma Chemical Co.
Sodium arsenate	arsenate	Fisher Scientific Co.
Glycine		Fisher Scientific Co.
Sodium Cacodylate		BDH Chemicals
Potassium pyroantimonate	pyroantimonate	Fisher Scientific Co.
^{124}Sb-pyroantimonate		New England Nuclear
Glutaraldehyde		Fisher Scientific Co. & Ladd Research Indus.
Osmium tetroxide	OsO_4	Johnson Matthey Chemicals Ltd.

Chemical	Abbreviation	Source
Disodium ethylenediamine tetraacetate	EDTA	Fisher Scientific Co.
Lanthanum nitrate	lanthanum	Fisher Scientific Co.
Iodoacetamide	IAAmide	Calibiochem
Adrenaline		Parke, Davis & Co., Ltd.
Oxytocin		Parke, Davis & Co., Ltd.
^{22}Na		Nuclear Science and Engineering
Epon 812	Epon	Polysciences, Inc.
Diethylstilbesterol		Merck Sharp & Dohme
Luciferase-Luciferin enzyme extract	Firefly Extract	Sigma Chemical Co.